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(71) Applicant : **EUROPEAN ECONOMIC COMMUNITY E.E.C.**
Bâtiment Jean Monnet
Plateau du Kirchberg
L-2920 Luxembourg (LU)

(72) Inventor : **Klein,Jürgen Robert**
Mackenbacher Strasse 15
D-67685 Weilerbach (DE)
Inventor : **Plapp,Roland**
Am Harzhübel 88
D-67663 Kaiserslautern (DE)

(74) Representative : **Grosset-Fournier, Chantal Catherine**
Grosset-Fournier & Demachy s.a.r.l.
103 rue La Fayette
F-75010 Paris (FR)

(54) Lys-aminopeptidase PepN from lactobacillus delbruckii ssp. Lactis, nucleic acids coding for it, and its use in fermentation processes.

(57) The invention relates to a protein as produced by *Lactobacillus delbrückii* ssp. *lactis*, and capable of hydrolysing β-naphtylamides, or fragments thereof having this enzymatic activity.
The invention relates also to the use of said protein or fragments thereof for the preparation of fermented foodstuff, and more particularly of cheese.

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The invention relates to a protein, PepN, as produced by *Lactobacillus delbrückii* ssp. *lactis* and capable of hydrolysing β-naphtylamides, or polypeptides derived thereof, and more particularly recombinant PepN from said *Lactobacillus* strain or recombinant polypeptides derived thereof, said derived polypeptides having this enzymatic activity of hydrolysing β-naphtylamides.

The invention relates also to the use of said protein or polypeptides derived thereof in fermentation processes, such as processes for the preparation of fermented foodstuff, and more particularly cheese.

The invention also relates to processes for preparing the PepN from said *Lactobacillus* strain or said derived polypeptides, which are in a state of biological purity such that they can be used in fermentation processes.

The invention also relates to nucleic acids (or nucleotide sequences) coding for PepN from said *Lactobacillus* strain or said derived polypeptides, and to cellular hosts containing said nucleic acids and their use in fermentation processes.

Furthermore, the invention relates to fermentation processes, and more particularly to processes for the preparation of fermented foodstuff, and kits, using the PepN from said *Lactobacillus* strain or said derived polypeptides, and/or said cellular hosts.

By "recombinant polypeptides" it is to be understood that it relates to any molecule having a polypeptidic chain liable to be produced by genetic engineering, through transcription and translation of a corresponding DNA sequence under the control of appropriate regulation elements within a efficient cellular host. Consequently the expression "recombinant polypeptides" such as is used herein does not exclude the possibility for the polypeptides to comprise other groups, such as glycosylated groups.

The term "recombinant" indeed involves the fact that the polypeptide has been produced by genetic engineering, particularly because it results from the expression in a cellular host of the corresponding nucleic acid sequences which have previously been introduced into the expression vector used in said host.

Nevertheless, it must be understood that this expression does not exclude the possibility for the polypeptide to be produced by a different process, for instance by classical chemical synthesis according to methods used in the protein synthesis or by proteolytic cleavage of larger molecules.

The expression "biologically pure" or "biological purity" or "in a substantially pure form" means on the one hand a grade of purity such that the polypeptide or recombinant polypeptide can be used for the preparation of fermented foodstuff and on the other hand the absence of contaminants, more particularly of natural contaminants.

The lactobacilli used as starter cultures in the industrial dairy fermentation need to have an efficient proteolytic system. Since the concentration of free essential amino acids in milk are low, a variety of proteolytic enzymes is necessary for the breakdown of milk casein. By the combined action of proteinases and peptidases, milk protein is degraded to peptides and amino acids, which are required for cell growth and which contribute to the organoleptic properties of the foods. Also the flavour of milk products is primarily based on this degradation. The proteolytic cascade must be initiated extracellularly and successive coordinated degradation of peptides by endo- and exopeptidases, with cell wall and cytoplasmic localizations, generate amino acids necessary for growth. The importance of the proteolytic system for dairy product quality has resulted in an increased fundamental research of the enzymes and genes involved. Various genes and corresponding enzymes from lactococci strains have been investigated, but less is known about the proteolytic system of lactobacilli.

An aspect of the invention is to provide a new family of nucleic acids coding for new proteins and polypeptides which can be used in fermentation processes, and more particularly for the preparation of fermented foodstuff.

Another aspect of the invention is to provide cellular hosts transformed with said nucleic acids which can be used in fermentation processes, and more particularly as starter organisms in the fermentation of milk.

Another aspect of the invention is to provide nucleic acids coding for the peptidic chain of biologically pure recombinant polypeptides which enable their preparation on a large scale.

Another aspect of the invention is to provide new proteins and polypeptides which can be used in fermentation processes.

Another aspect of the invention is to provide new fermentation processes, such as processes for the preparation of fermented foodstuff, and more particularly cheese.

The invention relates to the cloning, expression, and nucleotide sequence of the lys-aminopeptidase gene (*pepN*) from *Lb. delbrückii* ssp. *lactis* and to some characteristics of the purified enzyme. The gene has been designated *pepN* since it complements an *E. coli pepN* mutation, and as reported hereafter, there is extensive amino acid homology to other aminopeptidases N (E.C. 3.4.11.2). The enzyme PepN is characterized by its specific action on β-naphtylamides.

The invention relates to a protein in a substantially pure form, as produced by *Lactobacillus delbrückii* ssp. *lactis*, said protein, also called PepN, being capable of hydrolysing β-naphtylamides, and more particularly ly-

sine-β-naphtylamide, or fragments thereof having this enzymatic activity (i.e. this hydrolysing amino acid β-naphtylamides activity).

By hydrolysing amino acid β-naphtylamides, one should understand that such activity corresponds to aminopeptidase N (PepN); see Table 3 hereafter.

The invention relates more particularly to the PepN protein such as described above and produced by *Lactobacillus delbrückii* ssp. *lactis* WS87, deposited at the Deutsche Sammlung von Mikroorganismen (DSM) under the number 7290 on October 15, 1992.

The invention relates more particularly to the PepN protein represented on figure 3, and by SEQ ID NO 2, or polypeptides derived thereof, such as fragments or muteins (which differ from said protein by addition and/or substitution and/or suppression of one or several amino acid) thereof, provided that said derived polypeptides are capable of hydrolysing β-naphtylamides.

Said PepN represented by SEQ ID NO 2, is more particularly characterized in that:

- its isoelectric point calculated from the nucleotide sequence represented by SEQ ID NO 1, is 4,48, and its isoelectric point determined by preparative isoelectric focusing after purification of said protein, is 4,2,
- its molecular weight calculated from the nucleotide sequence represented by SEQ ID NO 1, is 95,358 kDa, and its molecular weight determined after purification, is 95 kDa,
- its specific chromogenic substrates are chosen among those listed in Table 3,
- its specific inhibitors are the following:
  - . EDTA: end concentration 1 mM: 3 % relative activity,
  - . Phenanthroline: end concentration 1 mM: 4 % relative activity.

The percentages of relative activity indicated above, correspond to residual activities after addition of the inhibitor as compared with the control without inhibitor. The conditions and methods for determination are taken from "Proteolytic enzymes, a practical approach, Beynon R.J. and Bond J.S., Oxford University Press 1989." (Chapter 4, Determination of protease mechanism, Ben M. Dunn).

The PepN according to the invention is more particularly characterized in that it is obtained in a purified state, from a cell extract of said *Lactobacillus delbrückii* ssp. *lactis* WS87 by the following procedure:

- fractionation of the cell extract by salting out at 4°C with streptomycin sulfate,
- centrifugation,
- anion exchange chromatography by applying the supernatant obtained at the previous step to an appropriate column, such as a column of Q-Sepharose Fast Flow (Pharmacia), and pooling the eluted fractions having the highest specific activity against L-Pro-p-nitroanilide,
- preparative isoelectric focusing, by applying the pooled fractions obtained at the previous step to an appropriate column, such as a LKB column (Types 8100-1), with ampholytes within a range of pH 4 to 6, and pooling the eluted fractions having the highest specific activity against Lys-paranitroanilide.

The invention also relates to nucleic acids coding for a protein according to the invention, i.e. for the PepN protein or plypeptides derived thereof as described above.

The invention relates more particularly to nucleic acids characterized in that:

- they comprise all or part of the nucleic acid represented on figure 3, and by SEQ ID NO 1, coding for a protein according to the invention, or its complementary sequence,
- or they hybridize with all or part of said nucleic acid represented by SEQ ID NO 1, or with its complementary sequence.

The invention relates more particularly to a nucleic acid coding for the PepN protein represented by SEQ ID NO 2, or for polypeptides derived thereof having said hydrolysing β-naphtylamides activity, said nucleic acid comprising all or part of the nucleotide sequence delimited by the nucleotide located in position 316 and the nucleotide located in position 2844 in the nucleotide sequence represented by SEQ ID NO 1.

The invention also relates to any nucleic acid susceptible to hybridize with all or part of nucleic acids such as described above, and more particularly to any nucleic acid derived from this latter according to the degeneracy of the genetic code, and more particularly by substitution, and/or addition, and/or suppression of one or several nucleotides of said nucleic acids described above, said derived nucleic acid being able to code for the PepN protein represented by SEQ ID NO 2, or for polypeptides derived thereof such as defined above.

By way of illustration, the hybridization above-mentioned can be performed with membranes like Hybond™-N (Amersham) or Hybridization Transfer Membrane (Micron Separations Inc.) under conditions as described by the supplier.

The invention also relates to recombinant nucleic acid containing at least one of the nucleic acids according to the invention, inserted in a heterologous nucleic acid.

The invention also relates to recombinant vectors comprising a vector sequence, notably of the type plasmid (such as plasmids originating from lactic acid bacteria), cosmid or phage, and a nucleic acid described

above, in one of the non-essential sites for its replication, and optionally one or several nucleic acid(s) coding for other aminopeptidases such as PepX (Meyer-Barton et al., 1993).

Prefered recombinant vector contains in one of its non essential sites for its replication necessary elements to promote the expression of polypeptides according to the invention, in a cellular host and possibly a promoter recognized by the polymerase of the cellular host, particularly an inducible promoter and possibly a signal sequence and/or an anchoring sequence.

The invention relates more particularly to recombinant vector, such as described above, containing the elements enabling the expression by *E. coli* or lactic acid bacteria including *lactococcus* and, especially, lactobacilli of the thermophilic group, of a nucleic acid according to the invention, inserted in the vectors.

The invention relates more particularly to recombinant vector, such as described above, constructed from replicons isolated from lactobacilli, especially from thermophilic *Lactobacillus* species.

The invention also relates to cellular hosts which are transformed by a recombinant vector according to the invention, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for a protein according to the invention, and optionally, other aminopeptidases such as PepX, in these hosts.

The invention relates more particularly to cellular hosts, such as described above, chosen from among bacteria such as *E.coli*, or lactic acid bacteria including *lactococcus* and, especially, lactobacilli of the thermophilic group, transformed by a vector according to the invention.

From the nucleic acids of the invention, probes (i.e. cloned or synthetic oligonucleotides) can be inferred.

These probes can be from 25 nucleotides up to the total length of the *pepN* gene. These oligonucleotides can also be used as amplification primers in the PCR technique (PCR, Mullis and Faloona, Methods in Enzymology, vol. 155, p. 335, 1987) to generate specific enzymatically amplified fragments and/or as probes to detect fragments amplified between bracketing oligonucleotide primers.

The invention also relates to the expression products of a nucleic acid expressed by a transformed cellular host according to the invention.

The invention also relates to a process for preparing the PepN protein such as described above, comprising the following steps:

- the culture in an appropriate medium of a cellular host according to the invention,
- the recovery of the polypeptide produced by the above said cellular host from the above said culture medium,
- the purification of the protein thus obtained, more particularly according to the method described above.

The invention also relates to fermentation processes comprising a step of treatment of material to be fermented with:

- an appropriate amount of the PepN protein or polypeptides derived thereof according to the invention, and optionally other proteases, and more particularly aminopeptidases such as PepX, and/or
- an appropriate amount of at least one of the transformed cellular host such as described above, comprising a nucleotide sequence coding for PepN, and optionally for other proteases, and more particularly aminopeptidases such as PepX,
- and optionally, an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

The invention also relates to a process for the preparation of fermented foodstuff, and more particularly of cheese, which comprises a step of treatment of food material to be fermented, such as milk, with:

- an appropriate amount of the PepN protein polypeptides derived thereof according to the invention, and optionally other proteases, and more particularly aminopeptidases such as PepX,
- an appropriate amount of at least one of the transformed cellular host such as described above, comprising a nucleotide sequence coding for PepN, and optionally for other proteases, and more particularly aminopeptidases such as PepX,
- and optionally, an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

The process for the preparation of fermented foodstuff described above can also comprise a step of treatment of food material to be fermented, such as milk, with other species and strains susceptible to be used as starter organisms in fermentation processes, and more particularly lactic acid bacteria susceptible to produce a PepX protein, such as *Lactococcus*, *Streptococcus* and *Lactobacillus*.

The fermentation processes according to the invention, are more particularly characterized in that they can be used as fermentation processes for the obtention of hard cheeses, such as Emmentaler type cheese (South Germany, Switzerland).

The invention also relates to foodstuff, and more particularly cheeses, such as obtained by fermentation processes as described above.

The invention also relates to the use of all or part of nucleotide sequences described above, as tools for analytical purpose. In that respect, the invention relates more particularly to the use of the above-mentioned nucleotide sequences, and more particularly all or part of the SEQ ID NO 1 sequence, in procedures for amino

acid sequencing of peptides and production of chemical or pharmaceutical compounds in which the hydrolysis of β-naphtylamide residues is a required step.

The invention relates more particularly to the use of the polypeptides described above for the determination of the exact localization of PepN in *Lactobacillus delbrückii* ssp. *lactis*, rendering possible experiments performed by immunoblotting after cell fractionation and by electron microscopy of immunogold labelled peptidases.

The polypeptides of the invention can also be prepared according to the classical techniques in the field of peptide synthesis.

The synthesis can be carried out in homogeneous solution or in solid phase.

For instance, the synthesis technique in homogeneous solution which can be used is the one described by Houbenweyl in the book entitled "Methoden der organischen Chemie" (Method of organic chemistry) edited by E. Wünsch, vol. 15-I and II, THIEME, Stuttgart 1974.

The polypeptides of the invention can also be prepared in solid phase according to the methods described by Atherton and Sheppard in their book entitled "Solid phase peptide synthesis" (IRL Press, Oxford, New York, Tokyo, 1989).

The invention also relates to antibodies themselves formed against the PepN protein or polypeptides derived thereof according to the invention, more particularly by immunization of appropriate animals with said polypeptides and recovery of antibodies thus formed.

It goes without saying that this production is not limited to polyclonal antibodies.

It also relates to any monoclonal antibody produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or a rat, immunized against the purified polypeptide of the invention on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by its ability to produce the monoclonal antibodies recognizing the polypeptide which has been initially used for the immunization of the animals.

The invention also relates to any antibody of the invention labelled by an appropriate label of the enzymatic, fluorescent or radioactive type.

The invention also relates to the use of such antibodies, for example for the detection of the PepN protein or polypeptides derived thereof, as described above.

The invention also relates to a process for preparing the nucleic acids according to the invention.

A suitable method for chemically preparing the single-stranded nucleic acids (containing at most 100 nucleotides of the invention) can be carried out according to the automatic β-cyanoethyl phosphoramidite method of DNA synthesis described in Bioorganic Chemistry 4; 274-325 (1986).

In the case of single-stranded DNA, the material which is obtained at the end of the DNA synthesis can be used as such.

A suitable method for chemically preparing the double-stranded nucleic acids (containing at most 100 bp of the invention) comprises the following steps:

- DNA synthesis of one sense oligonucleotide using the automatic b-cyanoethyl phosphoramidite method above cited, and DNA synthesis of one antisense oligonucleotide using either the above-mentioned automatic b-cyanoethyl phosphoramidite method, or enzymatic transcription of the sense-strand using a specific primer hybridizing to the 3'-end of the sense strand,
- combining the sense and antisense oligonucleotide by hybridization in order to form a DNA duplex,
- cloning the DNA duplex obtained into a suitable plasmid vector and recovery of the DNA according to classical methods such as restriction enzyme digestion and agarose electrophoresis, or by PCR amplification according to the procedure outlined above.

A method for the chemical preparation of nucleic acids with lengths greater than 100 nucleotides - or base pairs, in the case of double-stranded nucleic acids - comprises the following steps:

- assembling the synthesized oligonucleotides, provided at their ends with different restriction sites, the sequences of which are compatible with the succession of amino acids in the natural peptide, according to the principle described by Urdea et al. in Proc. Nat. Acad. Sci. USA 80; 7461-7465 (1983),
- cloning the DNA thereby obtained into a suitable plasmid vector and recovery of the desired nucleic acid according to classical methods such as restriction enzyme digestion and agarose gel electrophoresis.

Other characteristics and advantages of the invention will appear in the following examples and the figures illustrating the invention.

In cell extracts of *Lactobacillus delbrückii* ssp. *lactis* DSM7290 a peptidase with the activity to hydrolyse phe-β-naphthylamide and his-β-naphthylamide could be detected. *Escherichia coli* lacking the enzyme activity in a enzymatic plate assay, which was based on the hydrolysis of these β-naphthylamide (β-NA) substrates, was used to screen high copy and low copy number plasmid libraries of size fractionated *Lactobacillus* DNA. Clones with the desired phenotype were detected, and the gene, designated *pepN*, was further subcloned and

sequenced. A large open reading frame of 2529 nt is predicted to encode a protein of 843 amino acids (95358 Da). Analysis of the *pepN* sequence indicated that the enzyme is not subjected to posttranslational modification or exported via processing of a signal peptide. Comparison of the *pepN* gene from *Lb. delbrückii* ssp. *lactis* DSM7290 indicates that it is homologous to genes of the family of Zn^{2+}-metallohydrolases and shows identity with the active centre Zn^{2+}-binding motif of these enzymes. Like in all other organisms the substrate lys-β-NA is more effectively cleaved than phe- or his-β-NA's, used for screening in *E. coli*. The cloned enzyme was extremely overexpressed in *E. coli* and subclonig of the gene in *Lactobacillus casei* resulted in a moderate overexpression of approximately 20-fold. The cloned enzyme was purified from the *pepN* deficient *E. coli* strain CM89, using the substrate lys-p-nitroanilide. In a four step procedure including streptomycin sulfate precipitation, anion exchange chromatography, and gelfiltration the peptidase was purified to electrophoretic homogeneity.

The structural proteinase genes (*prtP: Kok et al., 1988; Kiwaki et al., 1989; Vos et al., 1989a; prtM: Haandrickman et al., 1989; Kiwaki et al., 1989; Vos et al., 1989b;*), the cysteine aminopeptidase (*pepC,* Chapot-Chartier et al., 1993), the X-prolyl-dipeptidyl-aminopeptidase genes (*pepX*; Nardi et al., 1991, Mayo et al., 1991), and the general aminopeptidase *pepN* (Stroman, 1992; van Alen-Boerrigter et al., 1991;) of a number of lactococci strains have been cloned and sequenced. From *Lactobacillus* strains only the proteinase genes (Holck and Naes, 1992). From *Lb. paracasei* and the X-prolyl-dipeptidyl-aminopeptidase gene from *Lb. delbrückii* ssp. *lactis* DSM7290 (Meyer et al., 1993), have been cloned and sequenced. *Lb. delbrückii* ssp. *lactis* is used as starter culture in the fermentation of swiss type cheese. Strain DSM7290, originally designated WS87, isolated from Emmenthaler cheese was chosen for screening of peptidase genes.

It was screened for complementation and expression in *E. coli*, with subtrates cleaved in cell extracts of DSM7290, but reacting negative in the enzyme plate assay used for screening. It has been decided to screen for heterologous expression in *E. coli* because of still moderate transformation efficiencies in lactobacilli and since peptidase activities in lactobacillal colonies cannot be reliably detected as a reason of acidification of agar plates and rigidity of the cell envelope.

I) Material and methods

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used are summarized in Table 1. *Escherichia coli* was grown at 37°C in Luria broth (Miller, 1972), and lactobacilli in MRS (De Man et al., 1960) at 37°C. Ampicillin and kanamycin were added to concentrations of 100 and 40 μg/ml, respectively.

Transformations

Lb. casei LK1 (Zink et al., 1991) and *E. coli* cells (Dower et al., 1988) were transformed by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) as described before.

Recombinant DNA Techniques.

Restriction enzymes and other nucleic acid-modifying enzymes were used as recommended by the manufacturers. Isolation of plasmid DNA from *E. coli* was performed as described by Sambrock et al. (1989). Plasmid DNA isolation from *Lb. casei* or *Lb. curvatus* was performed as described elsewhere (Zink et al., 1991).

Isolation of chromosomal DNA from DSM7290

400 ml of prewarmed MRS medium was inoculated with 40 ml of an overnight culture of *Lb. delbrückii* ssp. *lactis* DSM7290 and incubated for 2.5 hours at 37°C. Cells were pelleted by centrifugation, washed once with 50 mM Tris-HCl pH 8, resuspended in 37,5 ml of sucrose 6.5 %, 50 mM Tris-HCl pH 8, 1 mM EDTA, 200 mg of Lysozym, and 2500 U of mutanolysin (Sigma) and incubated for 1 hour at 37°C. 3.75 ml of 0.25 M EDTA in 50 mM Tris-HCl pH 8 was added. Cells were lysed by the addition of 2.25 ml 20 % SDS. The lysed cells were subjected to proteinase K digestion at a concentration of 50 μg/ml, for 15 min at 50°C and 30 min at 60°C. EtBr was added to a concentration of 1 mg/ml and CsCl to reach a final densitity of 1.55 g/ml. Centrifugation to equilibrium and further purification of chromosomal DNA was performed as described by Sambrock et al. (1989).

Molecular cloning of the *Lb. delbrückii* ssp. *lactis pepN* gene

Construction of plasmid libraries:

Genomic plasmid libraries of *Lb. delbrückii* ssp. *lactis* DSM7290 were prepared in *E. coli* ER1562 using the positive selection vector pUH84 (Henrich and Plapp, 1984) and because overexpression of peptidase genes from this high copy number vector may be lethal to the host a second plasmid bank was constructed with vector pLG339 having only 6-8 copies per chromosome (Stoker et al., 1982). Partial *Sau*3A fragments of total DSM7290 DNA were size fractionated (10-40 % sucrose gradient) and 4-10 kb fragments were ligated into the dephosphorylated *Bam*HI sites of the vector molecules.

Identification of peptidase genes:

Colonies of *E. coli* transformed with the gene banks were screened for PepN activity by a plate staining method of Miller and Mackinnon (1974), recently used by Nardi et al. (1991). If chromogenic β-NA substrates are cleaved, reaction of the β-napthylamines with fast garnet CBC (Sigma), can be monitored by the formation of a red, non-diffusible azo dye.

DNA Sequence Analysis

For nucleotide sequencing of inserts in pLG339, a pair of universal sequencing primers, adjacent to the *Bam*HI cloning site was synthesized. Synthetic oligonucleotide primers deduced from the investigated sequence, were synthesized and allowed direct sequencing of double stranded plasmid DNA. The DNA sequence of each strand was determined using the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden), which is based on the dideoxynucleotide chain termination method (Sanger et al., 1977) in the presence of [α-^{35}S]dATP (Amersham). For computer-assisted sequence analysis the Microgenie (Beckman, Palo Alto, CA), PC-Gene (IntelliGenetics, East El Camino Real, CA), and HUSAR (GENIUSnet, Heidelberg) software were used.

Sequencing primers were synthesized using Applied Biosystems Model 392 DNA synthesizer and reagents.

Preparation of cell extracts

Cell pellets of *E. coli* from a 2 l overnight culture were washed with 50 mM Tris-HCl pH 8.0, pelleted by centrifugation and resuspended in 20 ml of the same buffer. The bacteria were sonicated on ice (Bandelin sonifier; Sonopuls HD60) until more than 90 % of the cells were broken. Cell debris were removed by centrifugation at 52,000 g and 4°C for 60 min.

Lactobacillal cell extracts were prepared with 50 ml cultures and the modification that prior sonification of the more rigid bacteria, glass beads (diameter, 0.17-0.18 mm) were added to the cell pellets (2 Vol glass-beads/ 1 Vol cells). Centrifugation was performed in a Hereus Biofuge RS28 at 51,000 g and 4°C for 60 min.

The supernatants of both preparations contained approximately 50 mg/ml of protein as determined by the method of Lowry (1951).

Enzyme assay and effects of various chemical reagents in crude cell extracts

For characterization of the enzyme the p-nitroanilide subtrates (Bachem) were dissolved in water and added to the reaction mixture [Tris-HCl 10 mM, pH 8.0, and varying amounts of purified enzyme (1-200 ng)] to a concentration of 1 mM in a volume of 250 µl. Release of p-nitroaniline was measured after a 10 min incubation at 37°C at 405 mm in a LKB Ultrospec plus spectrophotometer. For rapid screening of active fractions during purification of the enzyme, samples were prepared in micro titer plates and measured in a BIORAD model 2550 EIA reader.

To study the mechanism of enzyme action, the inhibitors phenylmethansulphonyl fluoride (PMSF) at a concentration of 1 mM, pepstatin A at 1 µg/ml, L-trans-epoxysuccinyl-leucylamide(4-guanidino)-butane (E-64) at 0.1 mM, and 1,10-phenanthroline or EDTA at 1 mM were added to the enzyme and incubated for 30 min at 37°C. The substrate lys-p-nitroanilide was added and activity was measured spectrophotometrically by the release of p-nitroaniline.

Isoelectric focusing

Isoelectric focusing was carried out in a LKB column (type 8100-1) with ampholytes (Serva) ranging from pH 4 to 6. The pH gradient was stabilized by a glycerol gradient.

Purification

Step 1: Streptomycin sulfate precipitation

At 4°C a solution of 10 % streptomycin sulfate was slowly added to cell extract while stirring, to reach a final concentration of 2 %. The precipitate was discarded after centrifugation (10 min, 20,000 g.)

Steps 2 and 3: Anion exchange chromatography

The supernatant of the streptomycin sulfate precipitation was concentrated by passage through a PM30 Diaflow membrane (Amicon) and applied to a 38 by 2.2 cm column of Q-sepharose fast flow (Pharmacia) equilibrated with 0.1 M NaCl in 20 mM Tris-HCl pH 8.0 at 4°C. Proteins were eluted with a linear NaCl gradient of 0 to 0.5 M NaCl respectively in the same buffer. In a second passage a gradient ranging form 0.4 to 0.7 M NaCl was chosen. 4 ml fractions were collected at a flow rate of 150 ml/h and those with high PepN activity were pooled and concentrated by passage through a PM30 Diaflow membrane.

Step 4: Gelfiltration.

The enriched PepN preparation from anion exchange chromatography was applied to a 55 by 2.2 cm column of Fractogel TSK HW-55(S) (Merck) equilibrated with 20 mM Tris-HCl pH 7.2 at 4°C. Elution was performed with the same buffer at 20 ml/min and 2 ml fractions were collected.

II) Results and Discussion

Cloning of the *Lb. delbrückii* ssp. *lactis* DSM7290 *pepN* gene.

The plasmid libraries of DSM7290 chromosomal DNA in pUH84 and pLG339 respectively were transformed into *E. coli* ER1562. The *pepN* gene could be isolated by screening the colonies for peptolytic activities using the chromogenic substrates his-β-NA and phe-β-NA. In the plate assay these substrates were not cleaved by the homologous PepN of the *E. coli* host strain, and additionally they were not cleaved by *E. coli* harbouring the recently cloned genes for *pepX* (X-prolyl-dipeptidyl aminopeptidase) (Meyer et al., 1993) and *pepP* (prolin iminopeptidase, unpublished) from *Lb. delbrückii* ssp. *lactis* DSM7290. The gene has been designated *pepN* since sequence analysis revealed extensive amino acid homology to other aminopeptidases N (E.C. 3.4.11.2).

From approximately 50,000 colonies harbouring high copy number genebank plasmids no positive reaction could be detected in the plate assay, but in transformants with the low copy number plasmid bank, with pLG339 as vector, 3 his-β-NA and 3 phe-β-NA cleaving colonies out of 21,000 could be detected. The inability to identify positive clones in the pUH84 genebank indicated that overexpression of the peptidase gene is lethal for *E. coli*. Restriction analysis revealed that all plasmids isolated had insert sizes ranging from 5.2 to 8.0 kb, with a conserved identical core region. This agreed with the observation that all plasmids coded for an enzyme cleaving both substrates his-β-NA and phe-β-NA.

The plasmid pJUK11 with the smallest insert size of 5.2 kb was subject for further experiments (FIG.1). The chromogenic β-NA substrates have been used for colony screening and the analogous p-nitroanilides for characterization of the enzyme.

Plasmid encoded proteins

E. coli cells harbouring pJUK11 were sonified and the crude cell extracts subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Laemmli, 1970). A major 95 kDa protein appeared in ER-1562(pJUK11) extracts which constituted at least 50 % of the cytoplasmic *E. coli* proteins (FIG.2). This remarkable high level expression of *pepN* might be the reason for its lethality if cloned in a high copy number plasmid.

Subcloning and nucleotide sequence analysis

Prior to sequence analysis the region coding for *pepN* was localized, calculating that approximately 2.6 kb should be sufficient to code for the 95 kDa protein detected by SDS-PAGE. Subcloning of a 3.2 kb *SphI/HindIII* fragment from pJUK11 in pUC18 (FIG.1) resulted in a plasmid coding for the *pepN* gene with expression of enzyme activity, but which was extremely unstable. Nevertheless it offered the possibility to determine the DNA sequence starting at the HindIII site with the universal primer of vector pUC18. The second strand, which is coding for *pepN* was sequenced using a synthesized 20mer primer, complementary to the tetracyclin resistance gene in pJUK11. The complete nucleotide sequence of both strands of the *SphI/HindIII* fragment was determined using synthetic oligonucleotide primers deduced from the investigated sequences. The lactobacillal nucleotide sequence determined (3122 bp, 49.4% C+G) contained one single open reading frame (FIG.3). Starting with a putative ATG codon it would code for 843 amino acids corresponding to a protein with a molecular weight of 95358 Da. This value corresponds well with the molecular weight of the enzyme predicted by SDS-PAGE.

Transcription of the *pepN* gene

Upstream of the ATG start codon spaced by six base pairs, a putative ribosome binding site with a hexanucleotide stretch (AGGAGG) complementary to the *E. coli* 16 S-rRNA, and sequences that resemble promoter -10 and -35 sequences (Fig. 3) elements of gram positives (Van de Guchte et al., 1992) can be located. Currently we are going to compare the functionality of these sequences in *E. coli* and *Lb. delbrückii* ssp. *lactis*. The *pepN* coding sequence is followed by an inverted repeat which may form a stem-loop structure with a dG of -12.8 kcal/mol, and which may function as a transcriptional terminator, as predicted by the computer algorithm of Brendel and Trifonov (1984).

Comparison of the lactobacillal PepN sequence to aminopeptidases of other species

Searching the EMBL database resulted in similarities to several lys-aminopeptidases (FIG.4) belonging to the aminopeptidase N family (E.C. 3.4.11.2). These enzymes, sharing a common pattern of primary structure in the part of their sequence involved in the binding of zinc, can be grouped together as a family of neutral zinc metallopeptidases. They catalyse the removal of N-terminal amino acids from peptides. The most significant homology is observed in a functionally important segment which contains the proposed Zn^{2+}-binding catalytic site (Jongeneel et al., 1989). The phylogenetic tree calculated (FIG.5) demonstrates the close relationship of the lactobacillal enzyme to those of the lactococcal species. The diversity in the N-terminal sequences is a result from signal sequences present in human, rat, and mouse enzymes. The absence of a signal peptide sequence and the hydrophilicity plot of the amino acid sequence according to Kyte and Doolittle (1982) which does not show transmembrane domains, indicate that PepN of *Lb. delbrückii* ssp. *lactis* might be a intercellular located enzyme. Until now, no structure allowing protein secretion is described for any peptidase of lactic acid bacteria, but since the action of peptidases is required for casein cleavage a yet unknown mechanism might be responsible for translocation to the cell surface.

Gene dosis effects

The knowledge of the *pepN* nucleotide sequence allowed subcloning of a minimal sized DNA fragment into different cloning vectors. As summarized in FIG.1 we succeeded in cloning a 2.9 kb DraI fragment of pJUK11 in both orientations into pLG339, thus indicating that the *pepN* promoter sequence is functional in *E. coli*. This fragment was also cloned into the medium copy number plasmid pBR322 and even into the high copy number plasmid pUC18. In the latter constructs only one of the two possible insert orientations were obtained, likely an effect of lethal overproduction, since *pepN* would then be under control of lac or tet promoters. The amount of PepN protein produced, increased with the copy number of the plasmids, but on costs of plasmid stability (FIG.6).

For homologous expression in a *Lactobacillus* starter culture the cloned peptidase gene was inserted in both orientations, as a *Sph*I-restriction fragment, into pJK355 (FIG.1), a *Lactobacillus* vector constructed from the cryptic plasmid from *Lb. curvatus* LTH683 (Klein et al., 1993) resulting in plasmids pJK361 and pJK362. In crude extracts of *Lb. casei* LK1, with a chromosomal *pepN* gene, a lys-pNA hydrolysing activity could be measured, but expression of *pepN* coded on plasmids pJK361 or pJK362 resulted in overproduction of enzyme visible in SDS-PAGE (FIG.3) and enzymatic activities in crude extracts were increased by 15 to 20 fold. This increase of PepN activity had no obvious effect on cell growth in MRS-broth, which could have been expected

due to an imbalance in peptide supply. The influence on growth in milk is still going to be investigated as well as the effects on changes in taste and texture of cheese produced with such a modified strain. Purified PepN of *Lc. lactis* is reported to have a debittering activity on tryptic digests of β-casein (Tan et al., 1992). If the cloned and overexpressed enzyme of DSM7290 has comparable activities this gene can be of great industrial interest.

Purification and partial characterization of the cloned peptidase N

Since PepN protein was extremely overexpressed in *E. coli* harbouring the plasmid coded gene, the enzyme could be purified to electrophoretic purity by use of a four-step procedure. Due to sequence homologies of the cloned lactobacillal *pepN* with the *Escherichia coli* aminopeptidase N gene, the enzymatic properties were investigated in CM89 a strain lacking the *E. coli* enzyme. CM89(pUK13) was chosen for purification because the plasmid was stable in the presence of Kanamycin and high yields of PepN protein were obtained. Table 2 summarizes the purification to electrophoretic purity by the use of streptomycin sulfate precipitation, anion exchange chromatographies on Q-sepharose, and gelfiltration on HW TSK 55(S). The final purification was approximately 11-fold with a recovery of 20 %. Estimated by SDS-PAGE and gelfiltration, the enzyme is a monomer with a molecular mass of 95 kDa, which agrees well with the value deduced from sequence analysis. The pI of 4.2, determined by isoelectric focusing agrees well with the predicted one of 4.48 deduced from amino acid sequence. Optimum PepN activity was measured at pH of 6.5 - 7.0 and temperatures of 45 - 55°C. Denaturation occurs at pH values below 4 and at temperatures above 60°C.

The classification based on the susceptibility to a group of protease inhibitors (PMSF, E-64, pepstatin, EDTA, and 1,10 phenanthroline) indicated that the enzyme belongs to the class of metallo proteases, as full inhibition with 1,10 phenanthroline or EDTA could be detected, whereas the inhibitors PMSF, Pepstatin A, or E-64 had no effect on enzyme activity. Additionally the homology to other aminopeptidases implies that PepN has a catalytic centre with zinc binding sites (FIG.4). The inhibition by both complexing agents could be specifically restored by addition of Mn^{2+} and Co^{2+}, while Zn^{2+} or Mg^{2+} had no effect. Therefore one might suspect that Mn^{2+} and Co^{2+} ions play an essential role in the hydrolytic mechanism.

The substrate specificity of PepN against various p-nitroanilides is shown in Table 3. The highest activity was found for lys-p-nitroanilide and a Lineweaver-Burk plot indicated a Km of 70 μM. In comparison with the enzyme of *Lc. lactis* ssp. *cremoris* (Tan and Konings, 1990), showing the closest sequence relationship (62 % similarity) the lactobacillal Km value demonstrates an 8-fold higher affinity against lys-pNA. There are certain similar properties such as specifity against several p-NA substrates with bulk or aliphatic N-terminal residues, and pH optimum. The enzyme differs in restoration of enzyme activity after 1,10 phenanthroline inhibition. The temperature optimum of 50°C and its stability at elevated temperatures, after a 20 min incubation at 60°C a residual activity of 33 % were measured, might be of interest for applications of the cloned enzyme in milk fermentations.

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Legends to the figures

FIG. 1. Circular map of plasmid pJUK11. The insert of chromosomal DNA of 5.2 kbp in size, is indicated as a shaded segment, with the *pepN* gene presented as dotted arrow. The linear expansion of the insert illustrates the sequencing strategy. The different restriction fragments used for subcloning into different vectors, all spanning the *pepN* gene, are indicated as black bars.

FIG. 2. Expression of PepN protein in *E. coli* (ER1562) and *Lb. casei* (LK1). Separation of crude cell extracts by SDS-PAGE (12 % acrylamide). Proteins were visualized by Coomassie staining. Lanes 1 and 2, molecular weight marker proteins; cell extracts of *E. coli* ER1562(pJUK11) [lane3] and ER1562(pLG339) [lane4]; cell extracts of LK1 [lane 7], LK1(pJK361) [lane5] and LK1(pJK362) [lane 6]. The position of PepN is marked by an arrow head.

FIG. 3. Nucleotide sequence of the *pepN* region and deduced amino acid sequence of PepN. The proposed -35 (212 to 234) and -10 (250 to 255) positions and ribosome binding site (305 to 309) are underlined. An inverted repeat (underlined at bps 2871 to 2891) with a dG value of -12.8 kcal/mol, followed by a run of T-residues, is proposed as a transcription terminator.

FIG. 4. Progressive alignment (TREE, software of HUSAR GENIUSnet, Heidelberg) of the protein sequence of PepN from *Lb. delbrückii* ssp. *lactis* DSM7290 with those from other aminopeptidases. Identical amino acids in the sequences are marked (*). The putative catalytic sites (A) with zinc-binding domains (Z) are indicated.

FIG. 5. Phylogenetic relationship of aminopeptidases from different species. The dendrogram was calcu-

lated from the progressive alignment of homologous protein sequences using the program TREE of HUSAR, which is based on the method described by Feng and Doolittle (1987). The vertical bars represent nucleotide changes in percent.

FIG. 6. Stability of different plasmids coding for *pepN* in *Escherichia coli*. ER1562 harbouring different plasmids (pJUK12, pJUK13, pJUK14, pJUK15) was grown in liquid medium with and without the appropriate antibiotic selection.

Table 1. Bacterial strains and plasmids

Strain		relevant genotype or phenotype	Reference or source
Lactobacillus casei	LK1 [a]		Zink et al., 1991
Lactobacillus delbrückii ssp. lactis	DSM7290 [b]	source of library, pepN$^+$	M. Busse [c]
Escherichia coli K-12	ER1562	EcoK r$^-$m$^+$, mcrA$^-$ mcrB$^-$	Raleigh et al. 1988, Biolabs
Escherichia coli K-12	CK89	pepN$^-$	Miller and Schwartz, 1978

Plas.	replicon, relevant feature	Reference or source
pUC18	ColE1, high copy number	Yanish-Peron et al., 1985
pUN84	ColE1, high copy number, positive selection for insert DNA	Henrich and Plapp, 1986
p8R322	ColE1, medium copy number	Sutcliffe, 1979
pLG339	pSC101, low copy number	Stoker et al. 1982
pUC355	pLC2, cryptic plasmid from Lb. curvatus LTH683	Klein et al. 1993
pUK11	pSC101 from pLG339	this work
pUK12	pSC101 from pLG339	this work
pUK13	pSC101 from pLG339	this work
pUK14	ColE1 from p8R322	this work
pUK15	ColE1 from pUC18	this work
pUC360	pLC2 from pUC355	this work
pUC361	pLC2 from pUC356	this work

[a] formerly referred to as VS97

[b] formerly referred to as VS87

[c] Weihenstephan, F.R.G.

Table 2. Purification of aminopeptidase N from *Lb. delbrückii* spp. *lactis* DSM7290

Purification step	Total activity[a] ($Ux10^6$)	Specific activity[b]	Yield (%)	Purification (fold)
Cell extract	2	4170	100	1
Streptomycin sulfate precipitation	1.96	4990	98	1.2
Q-Sepharose (0-0.5 M NaCl)	1.6	11350	80	2.7
Q-Sepahrose (0.4-0.7 M NaCl)	1.1	22920	55	5.5
TSK HW 55(S)	0.4	45980	20	11.0

[a] 1 Unit defined as realease of 1 μmole p-nitroaniline per minute at 37°C
[b] specific activity defined as units per mg of protein

Table 3. Relative activity of PepN for p-nitroanilide substrates

p-nitroanilide (-pNA) substrate	relative activity (%)
Lys-pNA	100
Leu-pNA	19
Ala-pNA	12
Phe-pNA	8
Pro-pNA	2
Tyr-pNA	2
Gly-pNA	1
Ala-Pro-pNA	0

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: COMMUNAUTE ECONOMIQUE EUROPEENNE
(B) STREET: BATIMENT JEAN MONNET - PLATEAU DU KIRCHBERG
(C) CITY: LUXEMBOURG
(D) STATE: LUXEMBOURG
(E) COUNTRY: LUXEMBOURG
(F) POSTAL CODE (ZIP): L2920

(ii) TITLE OF INVENTION: LYS-AMINOPEPTIDASE PepN FROM LACTOBACILLUS DELBRUCKII SSP. LACTIS, NUCLEIC ACIDS CODING FOR IT, AND ITS USE IN FERMENTATION PROCESSES

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 93-13586.1
(B) FILING DATE: 01-JUL-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3122 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 316..2844

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GATAGGGCTG AAATTATCAT TTTAAGCGCT TTAAATTAGC TATACAGATA AGTAACATTA 60

GTAACAATTG TCAAGAGACT GCAATAAAAG GAAAAGGCCA GCTGCTAGAC TGGTCTTTTA 120

CATATGCAAT TATTTCAAAA ATGGAATTAA TTTCCGTTAG AAACTGAATT GTCTGATCGA 180

GTTCAAGGGG CTGAGGTAGA CTGCAAACAG ATATTTTGCG TTAAATGGGC TTTATTTAGC 240

CTTTTTTGCT AGAATAGAGA AGTGTGAATA CAATATACGC GAGGAAAAAT CAGACGCGGA 300

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TTATAGGAGG AAACA ATG GCT GTT AAG CGT TTT TAC GAA ACA TTC CAT CCA      351
                 Met Ala Val Lys Arg Phe Tyr Glu Thr Phe His Pro
                   1               5                  10

GAT CAC TAC GAT CTA TAC ATC GAC GTT GAC CGG GCA GCA AGA TCT TTC      399
Asp His Tyr Asp Leu Tyr Ile Asp Val Asp Arg Ala Ala Arg Ser Phe
          15                  20                  25

TCA GGG ACT TCC ACC ATC CAT GGT GAA ATC CAG GAA GAA ACT GTC TTG      447
Ser Gly Thr Ser Thr Ile His Gly Glu Ile Gln Glu Glu Thr Val Leu
     30                  35                  40

GTC CAC CAA AAG TAC ATG ACC ATC AGC AAG GTC ACT GTT GAC GGC AAG      495
Val His Gln Lys Tyr Met Thr Ile Ser Lys Val Thr Val Asp Gly Lys
 45                  50                  55                  60

GAA GTG CCG TTT ACT TTT GGC GAC GAC TTT GAA GGC ATC AAG ATC GAA      543
Glu Val Pro Phe Thr Phe Gly Asp Asp Phe Glu Gly Ile Lys Ile Glu
                   65                  70                  75

GCC GGC AAG ACT GGG GAA GCG GTC ATC GCG ATC GAC TAC TCA GCG CCT      591
Ala Gly Lys Thr Gly Glu Ala Val Ile Ala Ile Asp Tyr Ser Ala Pro
              80                  85                  90

TTG ACT GAC ACT ATG ATG GGG ATC TAC CCG TCC TAC TAC CAA GTT GAT      639
Leu Thr Asp Thr Met Met Gly Ile Tyr Pro Ser Tyr Tyr Gln Val Asp
          95                  100                 105

GGC GTC AAG AAG GAA CTG ATC GGG ACC CAG TTT GAA ACT ACT TTT GCC      687
Gly Val Lys Lys Glu Leu Ile Gly Thr Gln Phe Glu Thr Thr Phe Ala
     110                 115                 120

CGG GAA GCC TTC CCA TGT GTT GAC GAA CCA GAA GCT AAG GCA ACC TTC      735
Arg Glu Ala Phe Pro Cys Val Asp Glu Pro Glu Ala Lys Ala Thr Phe
125                 130                 135                 140

TCC CTC GCC CTC AAG TTT GAC GAA CAC GAA GGC GAA ACT GTT TTG GCC      783
Ser Leu Ala Leu Lys Phe Asp Glu His Glu Gly Glu Thr Val Leu Ala
                145                 150                 155

AAC ATG CCA GAA GAC CGG GTA GAA AAC GGC GTG CAC TAC TTT AAG GAG      831
Asn Met Pro Glu Asp Arg Val Glu Asn Gly Val His Tyr Phe Lys Glu
            160                 165                 170

ACT GTC CGC ATG TCC AGC TAC CTG GTT GCT TTT GCC TTT GGT GAA ATG      879
Thr Val Arg Met Ser Ser Tyr Leu Val Ala Phe Ala Phe Gly Glu Met
        175                 180                 185

CGG TCA TTG ACC ACC CAC ACC AAG AGC GGG GTC TTG ATC GGG GTT TAC      927
Arg Ser Leu Thr Thr His Thr Lys Ser Gly Val Leu Ile Gly Val Tyr
    190                 195                 200

TCA ACT CAA GCC CAC ACT GAA AAG GAA CTG ACC TTC TCC TTG GAC ATT      975
Ser Thr Gln Ala His Thr Glu Lys Glu Leu Thr Phe Ser Leu Asp Ile
205                 210                 215                 220
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GCC AAG CGG GCA ATT GAA TTC TAC GAA GAC TTT TAC CAA ACT CCA TAT      1023
Ala Lys Arg Ala Ile Glu Phe Tyr Glu Asp Phe Tyr Gln Thr Pro Tyr
                225                 230                 235

CCG CTG CCG CAA TCA CTG CAG CTG GCC CTG CCT GAC TTC TCA GCC GGT      1071
Pro Leu Pro Gln Ser Leu Gln Leu Ala Leu Pro Asp Phe Ser Ala Gly
            240                 245                 250

GCC ATG GAA AAC TGG GGT CTG GTA ACC TAC CGG GAA GCC TAC CTG CTT      1119
Ala Met Glu Asn Trp Gly Leu Val Thr Tyr Arg Glu Ala Tyr Leu Leu
        255                 260                 265

TTG GAC CCG GAC AAC ACC ACT TTG GAA ATG AAG AAG CTG GTT GCG ACT      1167
Leu Asp Pro Asp Asn Thr Thr Leu Glu Met Lys Lys Leu Val Ala Thr
    270                 275                 280

GTG GTG ACC CAC GAA CTG GCC CAC CAA TGG TTC GGT GAC CTG GTA ACC      1215
Val Val Thr His Glu Leu Ala His Gln Trp Phe Gly Asp Leu Val Thr
285                 290                 295                 300

ATG GAA TGG TGG GAC AAC CTC TGG CTG AAC GAA AGT TTC GCC AAC ATG      1263
Met Glu Trp Trp Asp Asn Leu Trp Leu Asn Glu Ser Phe Ala Asn Met
                305                 310                 315

ATG GAA TAC CTG TCA GTT GAC CAC CTG GAA CCT AAC TGG CAC ATC TGG      1311
Met Glu Tyr Leu Ser Val Asp His Leu Glu Pro Asn Trp His Ile Trp
            320                 325                 330

GAA ATG TTC CAG ACT TCT GAA GCA GCG GCT GCC TTG ACC CGG GAT GCA      1359
Glu Met Phe Gln Thr Ser Glu Ala Ala Ala Ala Leu Thr Arg Asp Ala
        335                 340                 345

ACC GAC GGG GTA CAG TCA GTG CAC GTG GAA GTT AAT GAC CCG GCT GAA      1407
Thr Asp Gly Val Gln Ser Val His Val Glu Val Asn Asp Pro Ala Glu
    350                 355                 360

ATC GAC GCC CTC TTT GAC GGG GCC ATC GTT TAC GCC AAG GGG TCA AGA      1455
Ile Asp Ala Leu Phe Asp Gly Ala Ile Val Tyr Ala Lys Gly Ser Arg
365                 370                 375                 380

ATG CTG GTC ATG GTC CGG TCA CTT TTG GGC GAT GAA GCC TTG AGA AAG      1503
Met Leu Val Met Val Arg Ser Leu Leu Gly Asp Glu Ala Leu Arg Lys
                385                 390                 395

GGC TTG AAG CGC TAC TTT GAC AAG CAC AAG TTT GGC AAC GCG GCA GGT      1551
Gly Leu Lys Arg Tyr Phe Asp Lys His Lys Phe Gly Asn Ala Ala Gly
            400                 405                 410

GAC GAT CTC TGG GAT GCC TTG TCA ACG GCC ACT GAC TTG AAC ATT GGG      1599
Asp Asp Leu Trp Asp Ala Leu Ser Thr Ala Thr Asp Leu Asn Ile Gly
        415                 420                 425

GAA ATC ATG CAC ACT TGG CTG GAC CAG CCA GGC TAC CCA GTG GTG AAT      1647
Glu Ile Met His Thr Trp Leu Asp Gln Pro Gly Tyr Pro Val Val Asn
    430                 435                 440
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GCT TTT GTT GAG GAC GGC CAC TTG AAG CTG ACT CAG AAG CAA TTC TTC      1695
Ala Phe Val Glu Asp Gly His Leu Lys Leu Thr Gln Lys Gln Phe Phe
445                 450                 455                 460

ATC GGT GAA GGC AAG GAA GTC GGC CGC AAG TGG GAA ATT CCG CTT AAC      1743
Ile Gly Glu Gly Lys Glu Val Gly Arg Lys Trp Glu Ile Pro Leu Asn
                465                 470                 475

GCT AAC TTC AAG GCA CCG AAG ATC ATG TCA GAC GTT GAA CTT GAC CTG      1791
Ala Asn Phe Lys Ala Pro Lys Ile Met Ser Asp Val Glu Leu Asp Leu
            480                 485                 490

GGT GAC TAC CAG GCT CTG CGG GCA GAA GCC GGC CAC GCT CTG CGC TTG      1839
Gly Asp Tyr Gln Ala Leu Arg Ala Glu Ala Gly His Ala Leu Arg Leu
        495                 500                 505

AAC GTG GGC AAC AAC TCC CAC TTC ATC GTG AAG TAC GAC CAG ACT TTG      1887
Asn Val Gly Asn Asn Ser His Phe Ile Val Lys Tyr Asp Gln Thr Leu
    510                 515                 520

ATG GAC GAC ATC ATG AAG GAA GCC AAG GAC TTG GAT CCA GTT TCC CAA      1935
Met Asp Asp Ile Met Lys Glu Ala Lys Asp Leu Asp Pro Val Ser Gln
525                 530                 535                 540

TTG CAA TTG CTG CAA GAC CTG CGG CTT TTG GCA GAA GGC AAG CAG GCT      1983
Leu Gln Leu Leu Gln Asp Leu Arg Leu Leu Ala Glu Gly Lys Gln Ala
                545                 550                 555

TCA TAC GCT GAC GTG GTA CCA GTT CTG GAA CTC TTC AAG AAC TCA GAA      2031
Ser Tyr Ala Asp Val Val Pro Val Leu Glu Leu Phe Lys Asn Ser Glu
            560                 565                 570

AGC CAC ATT GTC AAC GAT GCT CTG TAC ACG ACT GCT GAT AAG CTG CGG      2079
Ser His Ile Val Asn Asp Ala Leu Tyr Thr Thr Ala Asp Lys Leu Arg
        575                 580                 585

CAA TTT GCC CCA GCC GGC AGT GAA GCT GAC AAG AAC CTG CGG GCT CTG      2127
Gln Phe Ala Pro Ala Gly Ser Glu Ala Asp Lys Asn Leu Arg Ala Leu
    590                 595                 600

TAC AAC GAC TTG TCC AAG GAC CAA GTT GCC CGT TTG GGC TGG CTG CCT      2175
Tyr Asn Asp Leu Ser Lys Asp Gln Val Ala Arg Leu Gly Trp Leu Pro
605                 610                 615                 620

AAG GCA GGG GAA AGC GAT GAA GAC ATT CAG ACC CGG CCA TAC GTT TTG      2223
Lys Ala Gly Glu Ser Asp Glu Asp Ile Gln Thr Arg Pro Tyr Val Leu
                625                 630                 635

TCT GCT AGC CTT TAC GGC CGC AAC GCT GAT TCA GAA AAG CAA GCC CAC      2271
Ser Ala Ser Leu Tyr Gly Arg Asn Ala Asp Ser Glu Lys Gln Ala His
            640                 645                 650

GAA ATC TAC GTG GAA TAC GCT GAT AAG TTG GCA GAA CTG TCC GCT GAT      2319
Glu Ile Tyr Val Glu Tyr Ala Asp Lys Leu Ala Glu Leu Ser Ala Asp
        655                 660                 665
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ATC CGG CCA TAC GTT TTG ATC AAC GAA GTT GAA AAC TAC GGG TCA AGC      2367
Ile Arg Pro Tyr Val Leu Ile Asn Glu Val Glu Asn Tyr Gly Ser Ser
    670                 675                 680

GAA TTG ACT GAC AAG CTG ATT GGT TTG TAC CAG GCA ACC AGT GAC CCA      2415
Glu Leu Thr Asp Lys Leu Ile Gly Leu Tyr Gln Ala Thr Ser Asp Pro
685                 690                 695                 700

TCA TTC AAG ATG GAC CTG GAA GCC GCG ATC GTG AAG AGC AAG GAC GAA      2463
Ser Phe Lys Met Asp Leu Glu Ala Ala Ile Val Lys Ser Lys Asp Glu
                705                 710                 715

GGC GAA CTG AAG AAG ATC GTT TCC TGG TTC AAA AAC GCT GAA ATC GTT      2511
Gly Glu Leu Lys Lys Ile Val Ser Trp Phe Lys Asn Ala Glu Ile Val
            720                 725                 730

AAG CCG CAG GAC TTG CGC GGC TGG TTC AGC GGC GTT TTG TCC AAC CCG      2559
Lys Pro Gln Asp Leu Arg Gly Trp Phe Ser Gly Val Leu Ser Asn Pro
        735                 740                 745

GCA GGT GAA CAG CTG GCC TGG GAC TGG ATC AGA GAC GAA TGG GCA TGG      2607
Ala Gly Glu Gln Leu Ala Trp Asp Trp Ile Arg Asp Glu Trp Ala Trp
    750                 755                 760

TTG GAA AAG ACG GTC GGC GGC GAC ATG GAA TTC GCT ACC TTC ATC ACT      2655
Leu Glu Lys Thr Val Gly Gly Asp Met Glu Phe Ala Thr Phe Ile Thr
765                 770                 775                 780

GTC ATC TCC CGC GTC TTC AAG ACC AAG GAA CGC TAC GAC GAA TAC AAC      2703
Val Ile Ser Arg Val Phe Lys Thr Lys Glu Arg Tyr Asp Glu Tyr Asn
                785                 790                 795

GCC TTC TTT ACT GAC AAG GAA AGC AAC ATG CTG CTG AAC CGG GAA ATC      2751
Ala Phe Phe Thr Asp Lys Glu Ser Asn Met Leu Leu Asn Arg Glu Ile
            800                 805                 810

AAG ATG GAC CGG AAG GTC ATC GCT AAC CGG GTA GAC TTG ATT GCC AGC      2799
Lys Met Asp Arg Lys Val Ile Ala Asn Arg Val Asp Leu Ile Ala Ser
        815                 820                 825

GAA CAA GCT GAC GTC AAC GCC GCG GTT GCT GCT GCT TTG CAA AAG          2844
Glu Gln Ala Asp Val Asn Ala Ala Val Ala Ala Ala Leu Gln Lys
    830                 835                 840

TAATTGAATA GAGCATAAGA AAACTGTTTC CGCTGAGAGC TGGAAACAGT TTTTTTATGT    2904

ATTCAACTTG TCTGCAATCG GTTACAATAT AGATGTAAAT ACTATCGTAC ATTCTTTGAG    2964

GTAATAAAAT GAACAACGAT TTTAAAGATA TCATGCAAAA CAGAAAGTCT ATCCGGCACT    3024

ATGATTCCAG CGTGAAGATT TCCCGTGACG AATTGCTGGA AATCATTAAT GAATCTATCT    3084

CTGCTCCAAG TGCCTGCAAC CTGCAGTCCT GGAAGCTT                            3122
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 843 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ala Val Lys Arg Phe Tyr Glu Thr Phe His Pro Asp His Tyr Asp
 1               5                  10                  15

Leu Tyr Ile Asp Val Asp Arg Ala Ala Arg Ser Phe Ser Gly Thr Ser
            20                  25                  30

Thr Ile His Gly Glu Ile Gln Glu Glu Thr Val Leu Val His Gln Lys
        35                  40                  45

Tyr Met Thr Ile Ser Lys Val Thr Val Asp Gly Lys Glu Val Pro Phe
    50                  55                  60

Thr Phe Gly Asp Asp Phe Glu Gly Ile Lys Ile Glu Ala Gly Lys Thr
65                  70                  75                  80

Gly Glu Ala Val Ile Ala Ile Asp Tyr Ser Ala Pro Leu Thr Asp Thr
                85                  90                  95

Met Met Gly Ile Tyr Pro Ser Tyr Tyr Gln Val Asp Gly Val Lys Lys
            100                 105                 110

Glu Leu Ile Gly Thr Gln Phe Glu Thr Thr Phe Ala Arg Glu Ala Phe
        115                 120                 125

Pro Cys Val Asp Glu Pro Glu Ala Lys Ala Thr Phe Ser Leu Ala Leu
    130                 135                 140

Lys Phe Asp Glu His Glu Gly Glu Thr Val Leu Ala Asn Met Pro Glu
145                 150                 155                 160

Asp Arg Val Glu Asn Gly Val His Tyr Phe Lys Glu Thr Val Arg Met
                165                 170                 175

Ser Ser Tyr Leu Val Ala Phe Ala Phe Gly Glu Met Arg Ser Leu Thr
            180                 185                 190

Thr His Thr Lys Ser Gly Val Leu Ile Gly Val Tyr Ser Thr Gln Ala
        195                 200                 205

His Thr Glu Lys Glu Leu Thr Phe Ser Leu Asp Ile Ala Lys Arg Ala
    210                 215                 220

Ile Glu Phe Tyr Glu Asp Phe Tyr Gln Thr Pro Tyr Pro Leu Pro Gln
225                 230                 235                 240

Ser Leu Gln Leu Ala Leu Pro Asp Phe Ser Ala Gly Ala Met Glu Asn
                245                 250                 255
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Trp Gly Leu Val Thr Tyr Arg Glu Ala Tyr Leu Leu Leu Asp Pro Asp
260 265 270

Asn Thr Thr Leu Glu Met Lys Lys Leu Val Ala Thr Val Val Thr His
275 280 285

Glu Leu Ala His Gln Trp Phe Gly Asp Leu Val Thr Met Glu Trp Trp
290 295 300

Asp Asn Leu Trp Leu Asn Glu Ser Phe Ala Asn Met Met Glu Tyr Leu
305 310 315 320

Ser Val Asp His Leu Glu Pro Asn Trp His Ile Trp Glu Met Phe Gln
325 330 335

Thr Ser Glu Ala Ala Ala Ala Leu Thr Arg Asp Ala Thr Asp Gly Val
340 345 350

Gln Ser Val His Val Glu Val Asn Asp Pro Ala Glu Ile Asp Ala Leu
355 360 365

Phe Asp Gly Ala Ile Val Tyr Ala Lys Gly Ser Arg Met Leu Val Met
370 375 380

Val Arg Ser Leu Leu Gly Asp Glu Ala Leu Arg Lys Gly Leu Lys Arg
385 390 395 400

Tyr Phe Asp Lys His Lys Phe Gly Asn Ala Ala Gly Asp Asp Leu Trp
405 410 415

Asp Ala Leu Ser Thr Ala Thr Asp Leu Asn Ile Gly Glu Ile Met His
420 425 430

Thr Trp Leu Asp Gln Pro Gly Tyr Pro Val Val Asn Ala Phe Val Glu
435 440 445

Asp Gly His Leu Lys Leu Thr Gln Lys Gln Phe Phe Ile Gly Glu Gly
450 455 460

Lys Glu Val Gly Arg Lys Trp Glu Ile Pro Leu Asn Ala Asn Phe Lys
465 470 475 480

Ala Pro Lys Ile Met Ser Asp Val Glu Leu Asp Leu Gly Asp Tyr Gln
485 490 495

Ala Leu Arg Ala Glu Ala Gly His Ala Leu Arg Leu Asn Val Gly Asn
500 505 510

Asn Ser His Phe Ile Val Lys Tyr Asp Gln Thr Leu Met Asp Asp Ile
515 520 525

Met Lys Glu Ala Lys Asp Leu Asp Pro Val Ser Gln Leu Gln Leu Leu
530 535 540

Gln Asp Leu Arg Leu Leu Ala Glu Gly Lys Gln Ala Ser Tyr Ala Asp
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```
Val Val Pro Val Leu Glu Leu Phe Lys Asn Ser Glu Ser His Ile Val
                565                 570                 575

Asn Asp Ala Leu Tyr Thr Thr Ala Asp Lys Leu Arg Gln Phe Ala Pro
            580                 585                 590

Ala Gly Ser Glu Ala Asp Lys Asn Leu Arg Ala Leu Tyr Asn Asp Leu
        595                 600                 605

Ser Lys Asp Gln Val Ala Arg Leu Gly Trp Leu Pro Lys Ala Gly Glu
    610                 615                 620

Ser Asp Glu Asp Ile Gln Thr Arg Pro Tyr Val Leu Ser Ala Ser Leu
625                 630                 635                 640

Tyr Gly Arg Asn Ala Asp Ser Glu Lys Gln Ala His Glu Ile Tyr Val
                645                 650                 655

Glu Tyr Ala Asp Lys Leu Ala Glu Leu Ser Ala Asp Ile Arg Pro Tyr
            660                 665                 670

Val Leu Ile Asn Glu Val Glu Asn Tyr Gly Ser Ser Glu Leu Thr Asp
        675                 680                 685

Lys Leu Ile Gly Leu Tyr Gln Ala Thr Ser Asp Pro Ser Phe Lys Met
    690                 695                 700

Asp Leu Glu Ala Ala Ile Val Lys Ser Lys Asp Glu Gly Glu Leu Lys
705                 710                 715                 720

Lys Ile Val Ser Trp Phe Lys Asn Ala Glu Ile Val Lys Pro Gln Asp
                725                 730                 735

Leu Arg Gly Trp Phe Ser Gly Val Leu Ser Asn Pro Ala Gly Glu Gln
            740                 745                 750

Leu Ala Trp Asp Trp Ile Arg Asp Glu Trp Ala Trp Leu Glu Lys Thr
        755                 760                 765

Val Gly Gly Asp Met Glu Phe Ala Thr Phe Ile Thr Val Ile Ser Arg
    770                 775                 780

Val Phe Lys Thr Lys Glu Arg Tyr Asp Glu Tyr Asn Ala Phe Phe Thr
785                 790                 795                 800

Asp Lys Glu Ser Asn Met Leu Leu Asn Arg Glu Ile Lys Met Asp Arg
                805                 810                 815

Lys Val Ile Ala Asn Arg Val Asp Leu Ile Ala Ser Glu Gln Ala Asp
            820                 825                 830

Val Asn Ala Ala Val Ala Ala Ala Leu Gln Lys
        835                 840
```

Claims

1. Protein in a substantially pure form, as produced and excreted by *Lactobacillus delbrückii* ssp. *lactis* and capable of hydrolysing aminoacid β-naphtylamides, and more particularly lysine-β-naphtylamide, or fragments thereof having this enzymatic activity.

2. Protein according to claim 1, such as produced by *Lactobacillus delbrückii* ssp. *lactis* WS87, deposited at the Deutsche Sammlung von Mikroorganismen (DSM) under the number 7290 on October 15, 1992.

3. Protein according to claim 1 or claim 2, characterized in that it corresponds to the one (also called PepN) represented on figure 3, and by SEQ ID NO 2, or fragments or muteins (which differ from said protein by addition and/or substitution and/or suppression of one or several amino acid) thereof, provided that said fragments and muteins are capable of hydrolysing β-naphtylamides.

4. Protein according to anyone of claims 1 to 3, characterized in that:
   - its isoelectric point calculated from the nucleotide sequence represented by SEQ ID NO 1, is 4,48, and its isoelectric point determined by preparative isoelectric focusing after purification of said protein, is 4,2,
   - its molecular weight calculated from the nucleotide sequence represented by SEQ ID NO 1, is 95,358 kDa, and its molecular weight determined after purification, is 95 kDa,
   - its specific inhibitors are the following:
     . EDTA: end concentration 1mM; 3 % relative activity,
     . phenanthroline: end concentration 1 mM: 4 % relative activity.

5. Protein according to anyone of claims 1 to 4, characterized in that it is obtained in a purified state, from a cell extract of said *Lactobacillus delbrückii* ssp. *lactis* WS87 by the following procedure:
   - fractionation of the cell extract by salting out at 4°C with streptomycin sulfate,
   - centrifugation,
   - anion exchange chromatography by applying the supernatant obtained at the previous step to an appropriate column, such as a column of Q-Sepharose Fast Flow (Pharmacia), and pooling the eluted fractions having the highest specific activity against L-Pro-p-nitroanilide,
   - preparative isoelectric focusing, by applying the pooled fractions obtained at the previous step to an appropriate column, such as a LKB column (Types 8100-1), with ampholytes within a range of pH 4 to 6, and pooling the eluted fractions having the highest specific activity against Lys-paranitroanilide.

6. Nucleic acid characterized in that it codes for a protein according to anyone of claims 1 to 5.

7. Nucleic acid according to claim 6, characterized in that:
   - it comprises all or part of the nucleic acid represented on figure 3, and by SEQ ID NO 1, coding for a protein according to anyone of claims 1 to 5, or its complementary sequence,
   - or it hybridizes with all or part of said nucleic acid represented by SEQ ID NO 1, or with its complementary sequence.

8. Nucleic acid according to claim 6 or claim 7, characterized in that it codes for the PepN protein represented by SEQ ID NO 2, or for polypeptides derived thereof having said hydrolysing β-naphtylamides activity, said nucleic acid comprising all or part of the nucleotide sequence delimited by the nucleotide located in position 316 and the nucleotide located in position 2844 in the nucleotide sequence represented by SEQ ID NO 1.

9. Recombinant nucleic acid containing at least one nucleic acid according to anyone of claims 6 to 8, inserted into a heterologous nucleic acid.

10. Recombinant vector comprising a vector sequence, notably of the type plasmid (such as plasmids originating from lactic acid bacteria), cosmid or phage, and a nucleic acid according to anyone of claims 6 to 8, in one of the non-essential sites for its replication, and optionally one or several nucleic acid(s) coding for other aminopeptidases such as PepX.

11. Cellular host which is transformed by a recombinant vector according to claim 10, and comprising the regulation elements enabling the expression of the nucleotide sequence coding for a protein according to

anyone of claims 1 to 5, and optionally, other aminopeptidases such as PepX, in this host.

12. Cellular host according to claim 12, chosen from among bacteria such as *E.coli*, or lactic acid bacteria including *lactococcus* and, especially, lactobacilli of the thermophilic group, transformed by a vector according to the invention.

13. Process for preparing a protein according to anyone of claims 1 to 5 comprising the following steps:
    - the culture in an appropriate medium of a cellular host according to claim 11 or 12,
    - the recovery of the polypeptide produced by the above said cellular host from the above said culture medium,
    - the purification of the protein thus obtained.

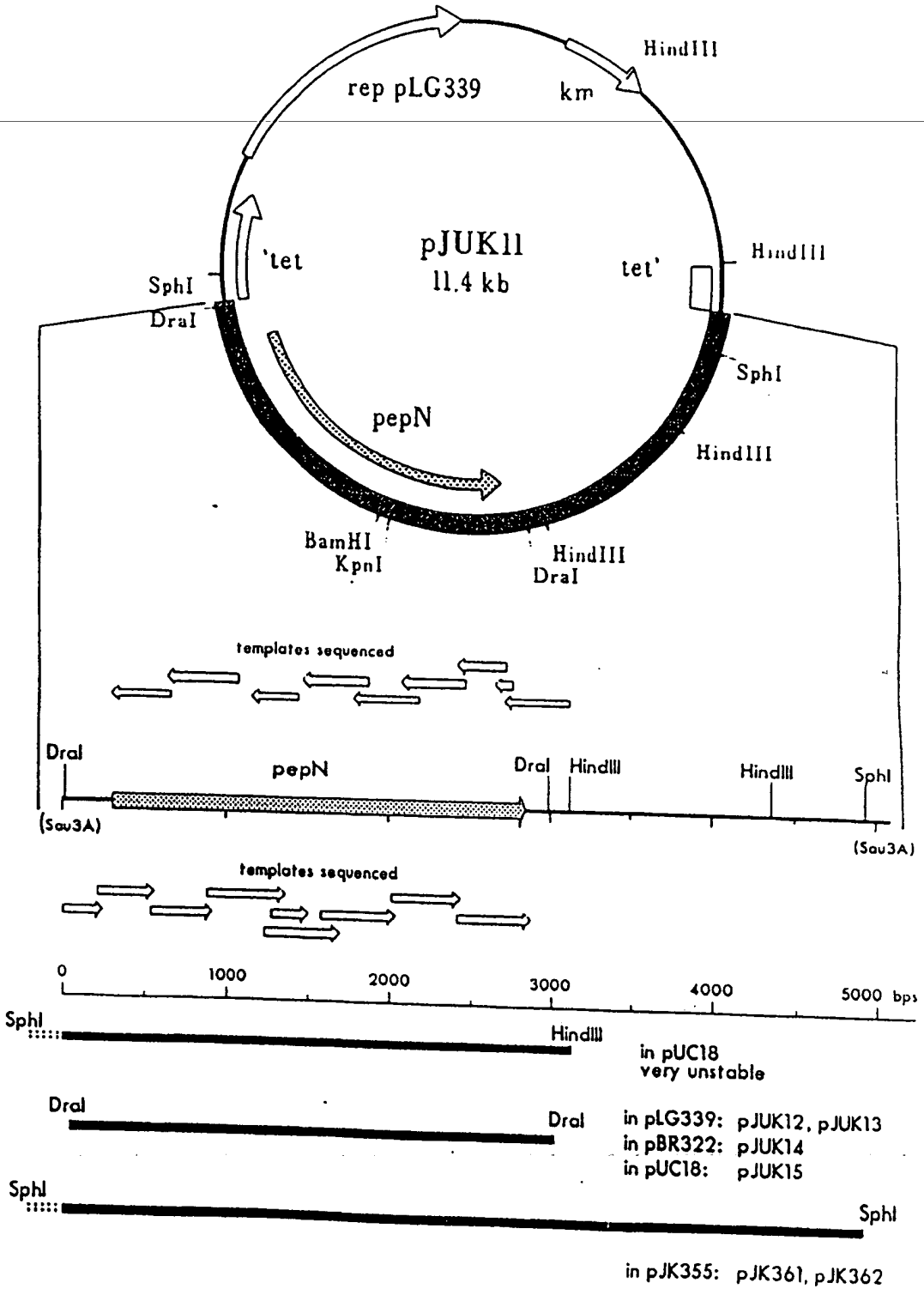
14. Fermentation process characterized in that it comprises a step of treatment of material to be fermented with:
    - an appropriate amount of the PepN protein or polypeptides derived thereof according to claims 1 to 5, and optionally other proteases, and more particularly aminopeptidases such as PepX, and/or
    - an appropriate amount of at least one of the transformed cellular host, according to claim 11 or 12,
    - and optionally, an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

15. Process for the preparation of fermented foodstuff, and more particularly of cheese, which comprises a step of treatment of food material to be fermented, such as milk, with:
    - an appropriate amount of at least one of the protein according to claims 1 to 5, and optionally other aminopeptidases such as PepX, and/or
    - an appropriate amount of at least one of the transformed cellular host according to of claim 11 or 12, and/or
    - an appropriate amount of lactobacilli, such as *Lactobacillus delbrückii* ssp. *lactis*.

16. Process according to claim 15, characterized in that it comprises a step of treatment of food material to be fermented, such as milk, with other species and strains susceptible to be used as starter organisms in fermentation processes, and more particularly lactic acid bacteria susceptible to produce a PepX protein, such as *Lactococcus*, *Streptococcus* and *Lactobacillus*.

17. Process according to claim 15 or claim 16, characterized in that it can be used as fermentation processes for the obtention of hard cheeses, such as Emmentaler type cheese

18. Foodstuff, and more particularly cheeses, such as obtained by fermentation processes, according to anyone of claims 15 to 17.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KpnI
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SphI
(Sau3A)
(Sau3A)
templates sequenced
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1000
2000
3000
4000
5000 bps
SphI
HindIII
in pUC18
very unstable
DraI
DraI
in pLG339: pJUK12, pJUK13
in pBR322: pJUK14
in pUC18: pJUK15
SphI
SphI
in pJK355: pJK361, pJK362

Figure 1

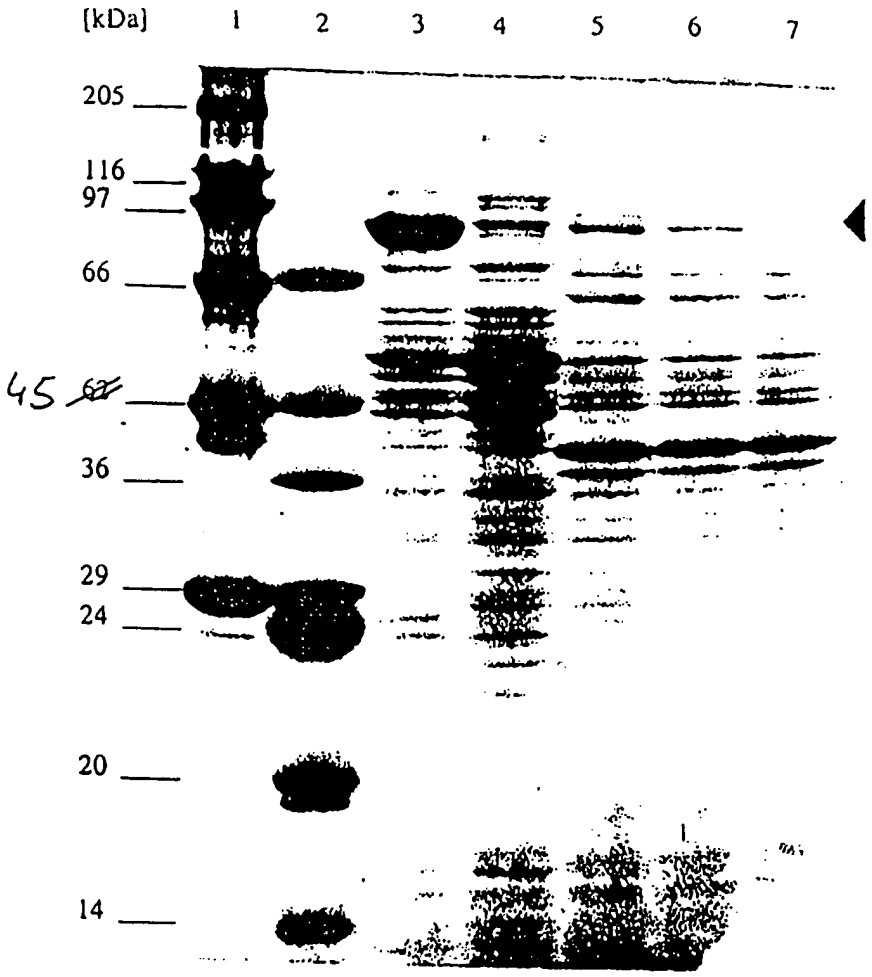

Figure 2

```
        10         20         30         40         50         60
GATAGGGCTGAAATTATCATTTTAAGCGCTTTAAATTAGCTATACAGATAAGTAACATTA

        70         80         90        100        110        120
GTAACAATTGTCAAGAGACTGCAATAAAAGGAAAAGGCCAGCTGCTAGACTGGTCTTTTA

       130        140        150        160        170        180
CATATGCAATTATTTCAAAAATGGAATTAATTTCCGTTAGAAACTGAATTGTCTGATCGA

       190        200        210        220        230        240
GTTCAAGGGGCTGAGGTAGACTGCAAACAGATATTTTGCGTTAAATGGGCTTTATTTAGC
                                          -35        proposed

       250        260        270        280        290        300
CTTTTTTGCTAGAATAGAGAAGTGTGAATACAATATACGCGAGGAAAAATCAGACGCGGA
promoter   -10

translation initiation      330        340        350        360
TTATAGGAGGAAACAATGGCTGTTAAGCGTTTTTACGAAACATTCCATCCAGATCACTAC
                MetAlaValLysArgPheTyrGluThrPheHisProAspHisTyr

       370        380        390        400        410        420
GATCTATACATCGACGTTGACCGGGCAGCAAGATCTTTCTCAGGGACTTCCACCATCCAT
AspLeuTyrIleAspValAspArgAlaAlaArgSerPheSerGlyThrSerThrIleHis

       430        440        450        460        470        480
GGTGAAATCCAGGAAGAAACTGTCTTGGTCCACCAAAAGTACATGACCATCAGCAAGGTC
GlyGluIleGlnGluGluThrValLeuValHisGlnLysTyrMetThrIleSerLysVal

       490        500        510        520        530        540
ACTGTTGACGGCAAGGAAGTGCCGTTTACTTTTGGCGACGACTTTGAAGGCATCAAGATC
ThrValAspGlyLysGluValProPheThrPheGlyAspAspPheGluGlyIleLysIle

       550        560        570        580        590        600
GAAGCCGGCAAGACTGGGGAAGCGGTCATCGCGATCGACTACTCAGCGCCTTTGACTGAC
GluAlaGlyLysThrGlyGluAlaValIleAlaIleAspTyrSerAlaProLeuThrAsp
```

Figure 3

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      610        620        630        640        650        660
ACTATGATGGGGATCTACCCGTCCTACTACCAAGTTGATGGCGTCAAGAAGGAACTGATC
ThrMetMetGlyIleTyrProSerTyrTyrGlnValAspGlyValLysLysGluLeuIle

      670        680        690        700        710        720
GGGACCCAGTTTGAAACTACTTTTGCCCGGGAAGCCTTCCCATGTGTTGACGAACCAGAA
GlyThrGlnPheGluThrThrPheAlaArgGluAlaPheProCysValAspGluProGlu

      730        740        750        760        770        780
GCTAAGGCAACCTTCTCCCTCGCCCTCAAGTTTGACGAACACGAAGGCGAAACTGTTTTG
AlaLysAlaThrPheSerLeuAlaLeuLysPheAspGluHisGluGlyGluThrValLeu

      790        800        810        820        830        840
GCCAACATGCCAGAAGACCGGGTAGAAAACGGCGTGCACTACTTTAAGGAGACTGTCCGC
AlaAsnMetProGluAspArgValGluAsnGlyValHisTyrPheLysGluThrValArg

      850        860        870        880        890        900
ATGTCCAGCTACCTGGTTGCTTTTGCCTTTGGTGAAATGCGGTCATTGACCACCCACACC
MetSerSerTyrLeuValAlaPheAlaPheGlyGluMetArgSerLeuThrThrHisThr

      910        920        930        940        950        960
AAGAGCGGGGTCTTGATCGGGGTTTACTCAACTCAAGCCCACACTGAAAAGGAACTGACC
LysSerGlyValLeuIleGlyValTyrSerThrGlnAlaHisThrGluLysGluLeuThr

      970        980        990       1000       1010       1020
TTCTCCTTGGACATTGCCAAGCGGGCAATTGAATTCTACGAAGACTTTTACCAAACTCCA
PheSerLeuAspIleAlaLysArgAlaIleGluPheTyrGluAspPheTyrGlnThrPro

     1030       1040       1050       1060       1070       1080
TATCCGCTGCCGCAATCACTGCAGCTGGCCCTGCCTGACTTCTCAGCCGGTGCCATGGAA
TyrProLeuProGlnSerLeuGlnLeuAlaLeuProAspPheSerAlaGlyAlaMetGlu

     1090       1100       1110       1120       1130       1140
AACTGGGGTCTGGTAACCTACCGGGAAGCCTACCTGCTTTTGGACCCGGACAACACCACT
AsnTrpGlyLeuValThrTyrArgGluAlaTyrLeuLeuLeuAspProAspAsnThrThr

     1150       1160   neutral Zn metallopeptidase signature
TTGGAAATGAAGAAGCTGGTTGCGACTGTGGTGACCCACGAACTGGCCCACCAATGGTTC
LeuGluMetLysLysLeuValAlaThrValValThrHisGluLeuAlaHisGlnTrpPhe
                                  Zn Act       Zn
```

Figure 3 (continued 1)

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      1210       1220       1230       1240       1250       1260
GGTGACCTGGTAACCATGGAATGGTGGGACAACCTCTGGCTGAACGAAAGTTTCGCCAAC
GlyAspLeuValThrMetGluTrpTrpAspAsnLeuTrpLeuAsnGluSerPheAlaAsn

      1270       1280       1290       1300       1310       1320
ATGATGGAATACCTGTCAGTTGACCACCTGGAACCTAACTGGCACATCTGGGAAATGTTC
MetMetGluTyrLeuSerValAspHisLeuGluProAsnTrpHisIleTrpGluMetPhe

      1330       1340       1350       1360       1370       1380
CAGACTTCTGAAGCAGCGGCTGCCTTGACCCGGGATGCAACCGACGGGGTACAGTCAGTG
GlnThrSerGluAlaAlaAlaAlaLeuThrArgAspAlaThrAspGlyValGlnSerVal

      1390       1400       1410       1420       1430       1440
CACGTGGAAGTTAATGACCCGGCTGAAATCGACGCCCTCTTTGACGGGGCCATCGTTTAC
HisValGluValAsnAspProAlaGluIleAspAlaLeuPheAspGlyAlaIleValTyr

      1450       1460       1470       1480       1490       1500
GCCAAGGGGTCAAGAATGCTGGTCATGGTCCGGTCACTTTTGGGCGATGAAGCCTTGAGA
AlaLysGlySerArgMetLeuValMetValArgSerLeuLeuGlyAspGluAlaLeuArg

      1510       1520       1530       1540       1550       1560
AAGGGCTTGAAGCGCTACTTTGACAAGCACAAGTTTGGCAACGCGGCAGGTGACGATCTC
LysGlyLeuLysArgTyrPheAspLysHisLysPheGlyAsnAlaAlaGlyAspAspLeu

      1570       1580       1590       1600       1610       1620
TGGGATGCCTTGTCAACGGCCACTGACTTGAACATTGGGGAAATCATGCACACTTGGCTG
TrpAspAlaLeuSerThrAlaThrAspLeuAsnIleGlyGluIleMetHisThrTrpLeu

      1630       1640       1650       1660       1670       1680
GACCAGCCAGGCTACCCAGTGGTGAATGCTTTTGTTGAGGACGGCCACTTGAAGCTGACT
AspGlnProGlyTyrProValValAsnAlaPheValGluAspGlyHisLeuLysLeuThr

      1690       1700       1710       1720       1730       1740
CAGAAGCAATTCTTCATCGGTGAAGGCAAGGAAGTCGGCCGCAAGTGGGAAATTCCGCTT
GlnLysGlnPhePheIleGlyGluGlyLysGluValGlyArgLysTrpGluIleProLeu

      1750       1760       1770       1780       1790       1800
AACGCTAACTTCAAGGCACCGAAGATCATGTCAGACGTTGAACTTGACCTGGGTGACTAC
AsnAlaAsnPheLysAlaProLysIleMetSerAspValGluLeuAspLeuGlyAspTyr
```

Figure 3 (continued 2)

```
      1810       1820       1830       1840       1850       1860
CAGGCTCTGCGGGCAGAAGCCGGCCACGCTCTGCGCTTGAACGTGGGCAACAACTCCCAC
GlnAlaLeuArgAlaGluAlaGlyHisAlaLeuArgLeuAsnValGlyAsnAsnSerHis

      1870       1880       1890       1900       1910       1920
TTCATCGTGAAGTACGACCAGACTTTGATGGACGACATCATGAAGGAAGCCAAGGACTTG
PheIleValLysTyrAspGlnThrLeuMetAspAspIleMetLysGluAlaLysAspLeu

      1930       1940       1950       1960       1970       1980
GATCCAGTTTCCCAATTGCAATTGCTGCAAGACCTGCGGCTTTTGGCAGAAGGCAAGCAG
AspProValSerGlnLeuGlnLeuLeuGlnAspLeuArgLeuLeuAlaGluGlyLysGln

      1990       2000       2010       2020       2030       2040
GCTTCATACGCTGACGTGGTACCAGTTCTGGAACTCTTCAAGAACTCAGAAAGCCACATT
AlaSerTyrAlaAspValValProValLeuGluLeuPheLysAsnSerGluSerHisIle

      2050       2060       2070       2080       2090       2100
GTCAACGATGCTCTGTACACGACTGCTGATAAGCTGCGGCAATTTGCCCCAGCCGGCAGT
ValAsnAspAlaLeuTyrThrThrAlaAspLysLeuArgGlnPheAlaProAlaGlySer

      2110       2120       2130       2140       2150       2160
GAAGCTGACAAGAACCTGCGGGCTCTGTACAACGACTTGTCCAAGGACCAAGTTGCCCGT
GluAlaAspLysAsnLeuArgAlaLeuTyrAsnAspLeuSerLysAspGlnValAlaArg

      2170       2180       2190       2200       2210       2220
TTGGGCTGGCTGCCTAAGGCAGGGGAAAGCGATGAAGACATTCAGACCCGGCCATACGTT
LeuGlyTrpLeuProLysAlaGlyGluSerAspGluAspIleGlnThrArgProTyrVal

      2230       2240       2250       2260       2270       2280
TTGTCTGCTAGCCTTTACGGCCGCAACGCTGATTCAGAAAAGCAAGCCCACGAAATCTAC
LeuSerAlaSerLeuTyrGlyArgAsnAlaAspSerGluLysGlnAlaHisGluIleTyr

      2290       2300       2310       2320       2330       2340
GTGGAATACGCTGATAAGTTGGCAGAACTGTCCGCTGATATCCGGCCATACGTTTTGATC
ValGluTyrAlaAspLysLeuAlaGluLeuSerAlaAspIleArgProTyrValLeuIle

      2350       2360       2370       2380       2390       2400
AACGAAGTTGAAAACTACGGGTCAAGCGAATTGACTGACAAGCTGATTGGTTTGTACCAG
AsnGluValGluAsnTyrGlySerSerGluLeuThrAspLysLeuIleGlyLeuTyrGln
```

Figure 3 (continued 3)

```
      2410        2420        2430        2440        2450        2460
GCAACCAGTGACCCATCATTCAAGATGGACCTGGAAGCCGCGATCGTGAAGAGCAAGGAC
AlaThrSerAspProSerPheLysMetAspLeuGluAlaAlaIleValLysSerLysAsp

      2470        2480        2490        2500        2510        2520
GAAGGCGAACTGAAGAAGATCGTTTCCTGGTTCAAAAACGCTGAAATCGTTAAGCCGCAG
GluGlyGluLeuLysLysIleValSerTrpPheLysAsnAlaGluIleValLysProGln

      2530        2540        2550        2560        2570        2580
GACTTGCGCGGCTGGTTCAGCGGCGTTTTGTCCAACCCGGCAGGTGAACAGCTGGCCTGG
AspLeuArgGlyTrpPheSerGlyValLeuSerAsnProAlaGlyGluGlnLeuAlaTrp

      2590        2600        2610        2620        2630        2640
GACTGGATCAGAGACGAATGGGCATGGTTGGAAAAGACGGTCGGCGGCGACATGGAATTC
AspTrpIleArgAspGluTrpAlaTrpLeuGluLysThrValGlyGlyAspMetGluPhe

      2650        2660        2670        2680        2690        2700
GCTACCTTCATCACTGTCATCTCCCGCGTCTTCAAGACCAAGGAACGCTACGACGAATAC
AlaThrPheIleThrValIleSerArgValPheLysThrLysGluArgTyrAspGluTyr

      2710        2720        2730        2740        2750        2760
AACGCCTTCTTTACTGACAAGGAAAGCAACATGCTGCTGAACCGGGAAATCAAGATGGAC
AsnAlaPhePheThrAspLysGluSerAsnMetLeuLeuAsnArgGluIleLysMetAsp

      2770        2780        2790        2800        2810        2820
CGGAAGGTCATCGCTAACCGGGTAGACTTGATTGCCAGCGAACAAGCTGACGTCAACGCC
ArgLysValIleAlaAsnArgValAspLeuIleAlaSerGluGlnAlaAspValAsnAla

      2830        2840        2850          proposed transcription
GCGGTTGCTGCTGCTTTGCAAAAGTAATTGAATAGAGCATAAGAAAACTGTTTCCGCTGA
AlaValAlaAlaAlaLeuGlnLysEnd

      2890        2900        2910        2920        2930        2940
GAGCTGGAAACAGTTTTTTTATGTATTCAACTTGTCTGCAATCGGTTACAATATAGATGT
terminator

      2950        2960        2970        2980        2990        3000
AAATACTATCGTACATTCTTTGAGGTAATAAAATGAACAACGATTTTAAAGATATCATGC
```

Figure 3 (continued 4)

```
      3010       3020       3030       3040       3050       3060
AAAACAGAAAGTCTATCCGGCACTATGATTCCAGCGTGAAGATTTCCCGTGACGAATTGC

      3070       3080       3090       3100       3110       3120
TGGAAATCATTAATGAATCTATCTCTGCTCCAAGTGCCTGCAACCTGCAGTCCTGGAAGC

TT
```

Figure 3 (continued 5)

EMBL entry	ORGANISM	REFERENCE	HOMOLOGY in complete protein sequence (conserved amino acid changes permitted)	Abreviation in alignment
LLPEPN	Lc. lactis ssp. lactis MG1363	Tan et. al. 1992a	62 %	Lc. lactis
LLLYSAP	Lc. lactis ssp. cremoris Wg2	Strøman 1992	62 %	Lc. cremor
OCAMINO	rabbit	Noren et al. 1989	41 %	rabbit
HSAMPEPN	Homo sapiens	Olsen et al. 1988	45 %	man
M26710	rat	Helmiss 1989	44 %	rat
MMANTBP1	mouse	Wu et al.1990	44 %	mouse
SCAPE2G	Saccharomyces cerevisiae	Garcia-Alvarez et al. 1991	47 %	S. cerevis
ECPEPN	Escherichia coli	McCaman and Gabe 1986	39 %	E. coli

```
Lc. lactis                                                                                                    MAVKR    LIETFVPENYKIF LDIDRKTKK     IKGQVAIT
Lc. cremor                                                                                                    MAVKR    LIETFVPENYKIF LDIDRKTKK     IKGQVAIT
Lb. lactis                                                                                                    MAVKR    FYETFHPDNYDLY IDVDRAARS     FSGTSTIH
S. cerevis                                                                                                    MTSKTPNREILPDNVVPLHYDLT VEPDFKTFK     FEGSVKIE
man                                MAKGFYI SKSLGIL GILLGVAAVCTIIALSVVYSQEKNKNANSSPVASTTPSASATTNPASATTLDQSKAWNRYRLPNTLKPDSYQVT LRPYLTPNDRGLYVFKGSSTVR
rat                                MAKGFYI SKTLGIL GILLGVAAVCTIIALSVVYAQEKNRNAENSAIAPTLPGSTSATTSTTNPAIDESKPWNQYRLPKTLIPDSYQVT LRPYLTPNEQGLYIFKGSSTVR
rabbit
mouse                         MNFAEEEPSKKYCIKGKHVAIICGVVVAVGLIVGLSVGLTRSCEQDTTPAPSQPPPEASTALPPQDQNVCPDSEDESGEWKNFRLPDFINPVKYDLE VKALMEEDR     YTGIVTIS
E. coli                                                                                                       MTQQP  QAKYRHDYRAPDYQITDIDLTFDLDAQKTVVTAVSQAVR
```

```
Lc. lactis  GEAKD   TVVSFHTKGLHFNKVRAFSVDTNFIENEEDEEIV          VKIGETGRVT          VSFEYEAELTDNMHGIYPSYYE  VNGEKKNLIGT  QFESHFARQAFP
Lc. cremor  GEAKD   TVVAFHAKGLHFNKVRAFSVDTNFIENEEDEEIV          VKIGETGRVT          VSFEYEAELTDNMHGIYPSYYE  VNGEKKNLIGT  QFESHFARQAFP
Lb. lactis  GEIQE   ETVLVHQKYMTISKVTVDGKEVPFTFGDDFEGIK          IEAGKTGEAV          IAIDYSAPLTDTMHGIYPSYYQ  VDGVKKELIGT  QFETTFAREAFP
S. cerevis  LKINNPAIDTVTLNTVDTDIHSAKIGDVTSSEIISEEEQQVTT  FAFPKGTNSSFKGNAF          LDIKFTGILNDNMAGFYRAKYEDKLTGETKYMATT  QHEPTDARRAFP
man         FTCKEAT DVIIIHSKKLNYTLSQGHRVVLRGVGGSQPPDIDKTELVEPTEYLVVHLKGSLVK   DSQYEMDSEFEGELADDLAGFYRSEYM  EGNVRKVVATT  QHQAADARKSFP
rat         FTCNETT NVIIIHSKKLNYTNKGNHRVALRALGDTPAPNIDTTELVERTEYLVVHLQGSLVK   GHQYEMDSEFQGELADDLAGFYRSEYM  EGGNKKVVATT  QHQAADARKSFP
rabbit                                                                                                    QFQGELADDLAGFYRSEYM  EGNVRKVVATTQHQHQAADARKSFP
mouse       VNLSKPT RDLWLHIRETKITKLPE    LRRPSGEQVP IRRCFEYKKQEYVVIQAAEDLAATSGDSVYRLTMEFKGWLNGSLVGFYKTTYM  EDGQIRSIAAT  DHEPTDARKSFP
E. coli     HGASD   APLRLNGEDLKLVSVHINDEPWTAVKEEEGALVI          SNLPERFTLK          IINEISPAAWTALEGLYQS       GDALCT   QCEAEGFRHITY
```

```
Lc. lactis  SIDEPEAKATFDLSVKFDE EEGDIIVSNMPE    LLNING  IHVFERTVKMSSYLLAFVFGELQYKKG  KTKSGVEVGAFATKAH SQAALDFPLDIAIRSIEFYEDYYQTPYPLP
Lc. cremor  SIDEPEAKATFDLSVKFDE EEGDIIVSNMPE    LLNING  IHVFERTVKMSSYLLAFVFGELQYKKG  KTKSGVEVGAFATKAH SQAALDFPLDIAIRSIEFYEDYYQTPYPLP
Lb. lactis  CVDEPEAKATFSLALKFDE HEGETVLAKMPE    DRVENG  VHYFKETVRMSSYLVAFAFGEMRSLTT  HTKSGVLIGVYSTQAH TEKELTFSLDIAKRAIEFYEDFYQTPYPLP
S. cerevis  CFDEPNLKASFAITLVSVP SLTHLSNMDVKN    EYVKDGKKVTLFNTTPKMSTYLVAFIVAELKYVES  K NFRIPVRVYATPGN EKHG QFAADLTAKTLAFFEKTFGIQYPLP
man         CFDEPAMKAEFNITLIHPK DLTALSNMLPKGPSTPLPEDPNWNVTEFHTTPKMSTYLLAFIVSEFDYVEK  QASNGVLIRIWARPSAIAAGHGDYALNVTGPILNFFAGHYDTPYPLP
rat         CFDEPAMKASFNITLIHPN NLTALSNMLPKDSRT LQEDPSWNVTEFHPTPKMSTYLLAYIVSEFKYVEA  VSPNRVQIRIWARPSAIDEGHGDYALQVTGPILNFFAQHYNTAYPLE
rabbit      CFDEPASKATFNITLIHPR DYTALSNMLPRSSTA LPEDPNWTVTEFHTTPKMSTYLLAYIVSEFTNIEA  QSPNNVQIRIWARPSAISEGHGQYALNVTGPILNFFANHYNTPYPLE
mouse       CFDEPNKKSTYSISIIHPK EYSALSNM PEEKSE MVDD NVKKTTFVKSVPMSTYLVCFAVHRFTAIER  KSRSGKPLKVYVQPN  QKETAEYAANITQAVFDYFEDYFAMEYALP
E. coli     YLDRPDVLARFTTKIIADKIKYPFLLSNGNRVAQGELENGRHW  VQWQDPFPKPCYLFALVAGDFDVLRDTFTTRSGREVALELYVDRGNLDRAPWAMTSLKNSMKWDEERFGLEYDLD
```

```
Lc. lactis  HSWHIALPDFSAGAMENWGCITYREVCMLVDPENATIQSKQYVATVIAHELAHQWFGDLVTMQWWDDLWLNESFANNMEYVCMDALEPSWNVWESFSISEANMALNRDATDGVQSV  H
Lc. cremor  HSWHIALPDFSSGAMENWGCITYREVCMLVDPENATIQSKQYVATVIAHELAHQWFGDLVTMQWWDDLWLNESFANNMEYVCMDALEPSWNVWESFSISEANMALNRDATDGVQSV  H
Lb. lactis  QSLQLALPDFSAGAMENWGLVTYREAYLLLDPDNTTLEMKKLVATVVTHELAHQWFGDLVTMEWWDNLWLNESFANMMEYLSVDHLEPNWHIWEMFQTSEAAAALTRDATDGVQSV  H
S. cerevis  KHDNVAVHEFSAGAMENWGLVTYRVVDLLLDKDNSTLDRIQRVAEVVQHELAHQWFGNLVTMDWWEGLWLNEGFATWMSWYSCNEFQPEWKVWEQYVTDTLQHALSLDSLRSSHPI  E
man         KSDQIGLPDFNAGAMENWGLVTYRENSLLFDPLSSSSSNKERVVTVIAHELAHQWFGNLVTIEWWNDLWLNEGFASYVEYLGADYAEPTWNLKDLMVLNDVYRVHAVDALASSHPLSTPA
rat         KSDQIALPDFNAGAMENWGLVTYRESALVFDPOSSSISNKERVVTVIAHELAHQWFGNLVTVDWWNDLWLNEGFASYVEYLGADYAEPTWNLKDLIVLNDVYRVHAVDALASSHPLSSPA
rabbit      KSDQIGLPDFNAGAMENWGLVTYRESALLFDPLVSSISNKERVVTVVAHELAHQWFGNLVTVDWWNDLWLNEGFASYVEFLGADYAEPTWNLKDLIVLNDVYRVHAVDALASSHPLSSPA
mouse       KLDKIAIPDFGTGAMENWGLVTYRETNLLYDPLLSASSNQQRVASVVAHELVHQWFGNTVTMDWWDDLWLNEGFASFFEFLGVNHAEKDWQMLSQVLLEDVFPVQEDDSLMSSHPV  V
E. coli     IYMIVAVDFFNMGAMENKGLNIFNSKYVLARTDTATDKDYLDIERVIGHEYFHNWTGNRVTCRDWFQLSLKEGLTVFRD    QEFSSDLGSRAVNRINNVRTMRGLQFAEDASPM AHP
                                                                                                                  :::ZA::Z:::
```

Figure 4

EP 0 633 316 A1

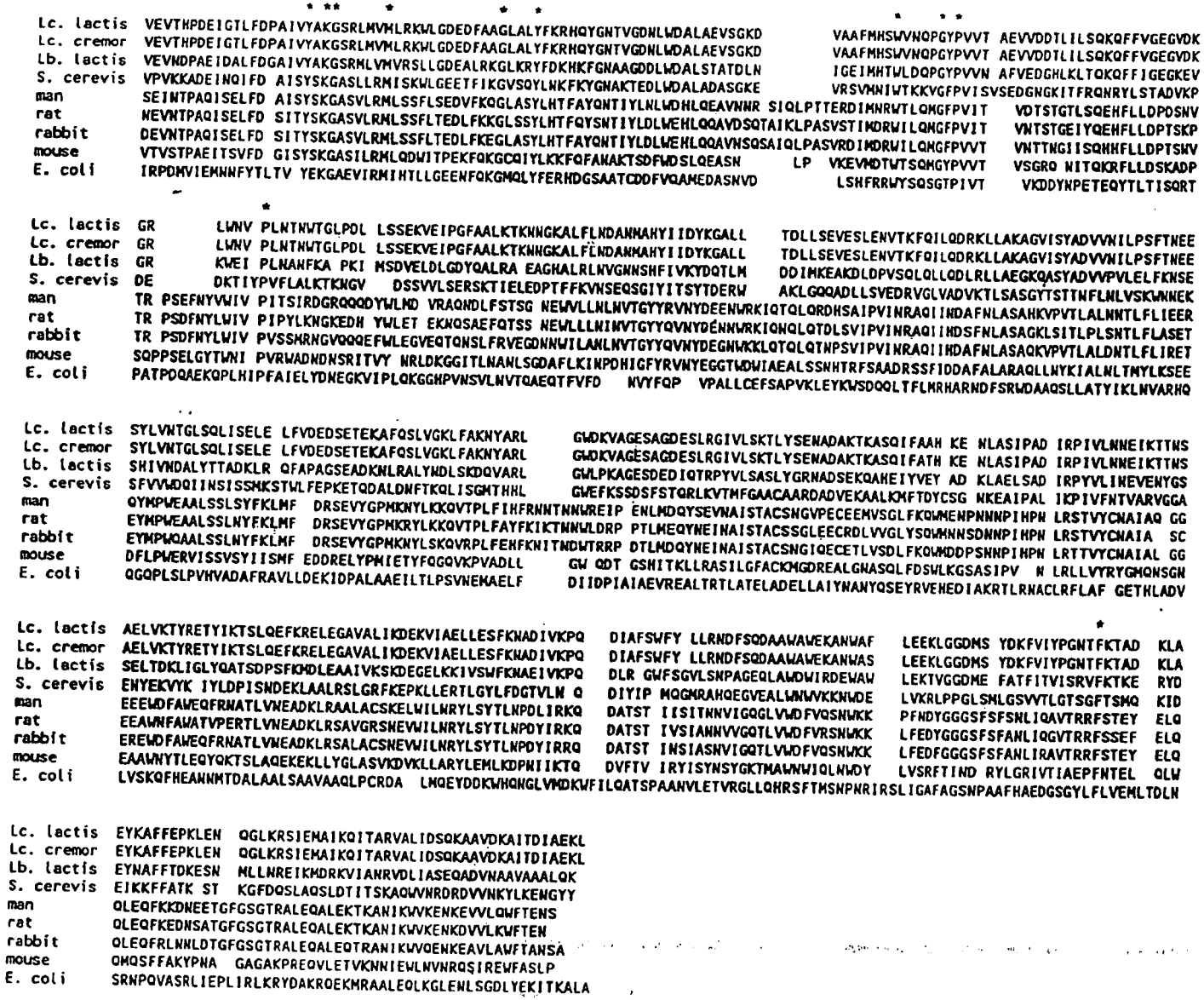

Figure 4 (continued 1)

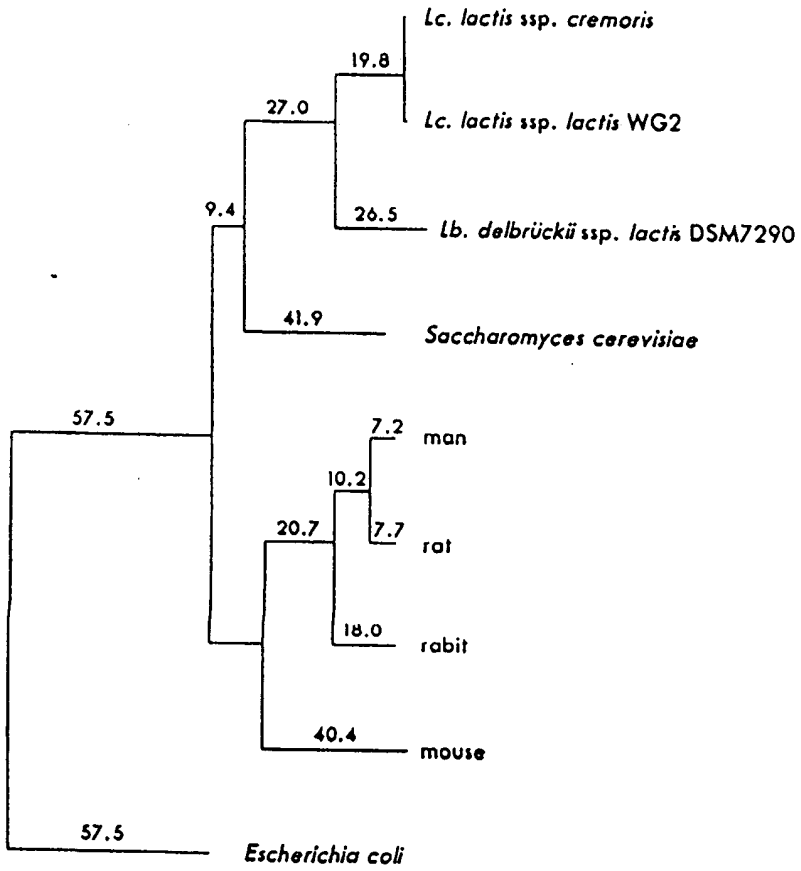
Lc. lactis ssp. cremoris
19.8
27.0
Lc. lactis ssp. lactis WG2
9.4
26.5
Lb. delbrückii ssp. lactis DSM7290
41.9
Saccharomyces cerevisiae
57.5
7.2
man
10.2
20.7
7.7
rat
18.0
rabit
40.4
mouse
57.5
Escherichia coli

Figure 5

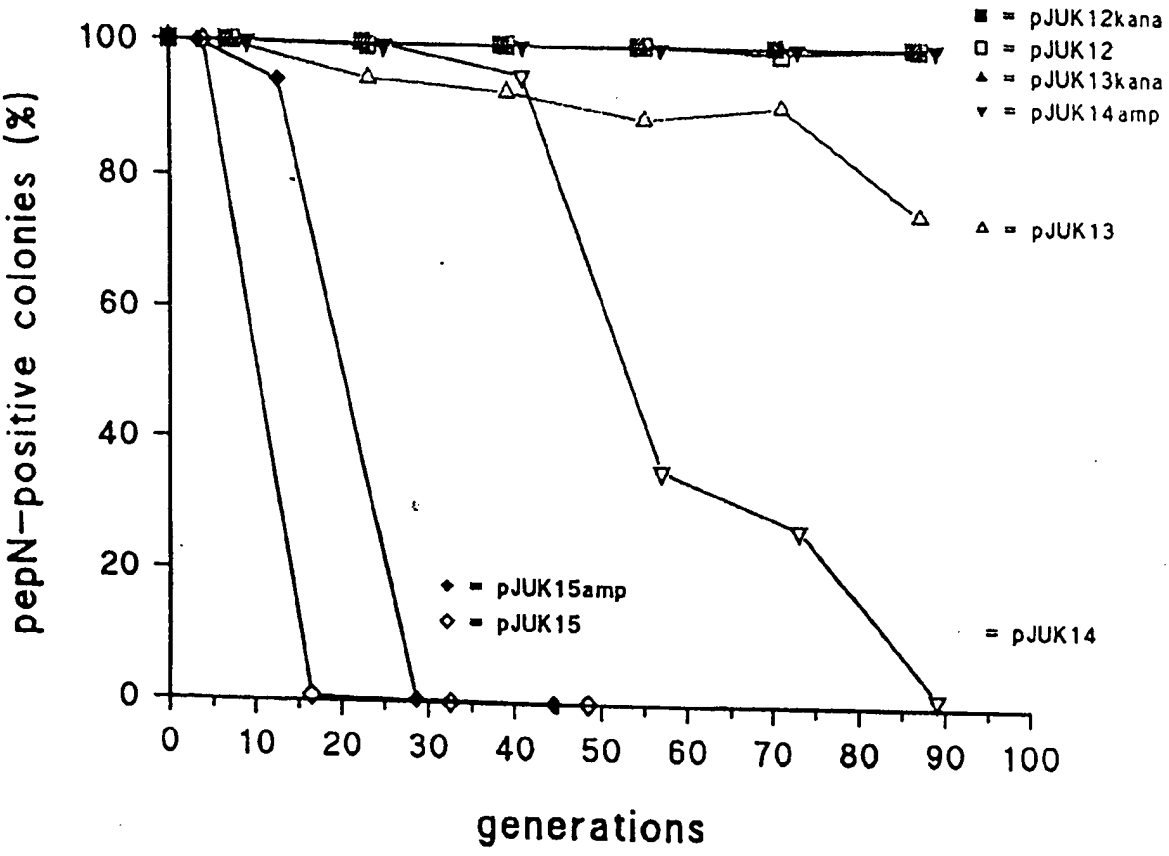
pepN-positive colonies (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
generations
■ = pJUK12kana
□ = pJUK12
▲ = pJUK13kana
▼ = pJUK14amp
△ = pJUK13
◆ = pJUK15amp
◇ = pJUK15
= pJUK14

Figure 6

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 40 1497

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	JOURNAL OF DAIRY SCIENCE, vol.74, 1991, CHAPAIGN, ILLINOIS US pages 29 - 45 N. KHALID ET AL 'Peptide hydrolases of Lactobacillus helveticus and Lactobacillus delbrueckii ssp. bulgaricus' * page 36, left column - right column, paragraph 1; tables 1,5 * * abstract * ---	1-18	C12N15/57 C12N9/52 C12N1/21 A23C19/032 C12P21/06
Y	APPL. MICROBIOL. BIOTECHNOL., vol.39, May 1993 pages 204 - 210 C. NOMAKOWSKI ET AL 'Cloning of peptidase genes from Lactobacillus helveticus CNRZ 32' * the whole document * ---	1-18	
Y	GENE., vol.113, 1992, AMSTERDAM NL pages 107 - 112 P. STROMAN 'Sequence of a gene (lap) encoding a 95.3-kDa aminopeptidase from Lactococcus lactis ssp. cremoris Wg2' * the whole document * ---	1-18	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N A23C
Y	FEBS LETTERS., vol.306, no.1, July 1992, AMSTERDAM NL pages 9 - 16 P. TAN ET AL 'Characterization of the Lactococcus lactis pepN gene encoding an aminopeptidase homologous to mammalian aminopeptidase N' * the whole document * --- -/--	1-18	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	14 October 1994	Van der Schaal, C

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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EUROPEAN SEARCH REPORT

Application Number
EP 94 40 1497

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	INT. DAIRY JOURNAL, vol.1, 1991 pages 51 - 66 W. BOCKELMANN AND M. FOBKER 'Purification and characterization of the X-prolyl-dipeptidyl-aminopeptidase from Lactobacillus delbrueckii subsp. bulgaricus and Lactobacillus acidophilus' * abstract * ---	10,11, 14-18	
Y	EP-A-0 522 203 (PROBICOM RESEARCH B.V.) 13 January 1993 * abstract * ---	14-18	
P,Y	CHEMICAL ABSTRACTS, vol. 120, no. 19, 9 May 1994, Columbus, Ohio, US; abstract no. 237307, E. MEYER-BARTON ET AL 'Cloning and sequence analysis of the X-prolyl-dipeptidyl-aminopeptidase gene (pepX) from Lactobacillus delbrueckii ssp. lactis DSM7290' page 251 ; * abstract * & APPL. MICROBIOL. BIOTECHNOL., vol.40, no.1, October 1993 pages 82 - 89 --- -/--	1-18	TECHNICAL FIELDS SEARCHED (Int.Cl.6)

The present search report has been drawn up for all claims

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
P,Y	CHEMICAL ABSTRACTS, vol. 119, no. 21, 22 November 1993, Columbus, Ohio, US; abstract no. 220172, E. TSAKALIDOU ET AL 'A comparative study: aminopeptidase activities from Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus' page 407 ; * abstract * & JOURNAL OF DAIRY SCIENCE, vol.76, no.8, August 1993, CHAPAIGN, ILLINOIS US pages 2145 - 2151 ---	1-18	
P,X	EUR. J. BIOCHEMISTRY, vol.217, October 1993 pages 105 - 114 J. KLEIN ET AL 'Cloning, DNA sequence analysis and partial characterization of pepN, a lysyl aminopeptidase from Lactobacillus delbrückii ssp. lactis DSM7290' * the whole document * -----	1-18	TECHNICAL FIELDS SEARCHED (Int.Cl.6)

The present search report has been drawn up for all claims

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